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(54) Title: A METHOD OF IDENTIFYING AND RECOVERING PRODUCTS EXUDED FROM A PLANT

(57) Abstract

This invention provides a method of identifying biologically active or otherwise valuable substances exuded from or onto a plant surface, specifically the plant cuticle. This invention also provides a method of identifying and recovering substances exuded from or onto the roots of a plant. The invention further comprises libraries of substances exuded or secreted from various plant species, which may be elicited or induced to produce one or more of such substances.

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A METHOD OF IDENTIFYING AND RECOVERING PRODUCTS EXUDED FROM A PLANT

This application claims priority to U.S. Application No. 09/130,185, filed August 6, 1999, the entirety of which is incorporated by reference herein. This application is related to commonly-owned, co-pending U.S. Application Serial Nos. 09/067,836, filed April 28, 1998, and 09/203,772, filed June 23, 1998, the entireties of which are incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates to a method of identifying and recovering biologically active agents that are exuded from or onto a plant or plant part. The methods disclosed herein will aid in the discovery of new agents, compounds or drugs having diverse biological activities and properties, for treatments of various diseases or conditions, as well as serving as flavors, fragrances, and additives.

BACKGROUND OF THE INVENTION

Plants are recognized as being a potential source of chemical compounds (phytochemicals) having biological activity or other properties of interest to the medical, agricultural and food processing communities, among others. A variety of such compounds have been isolated from plants and used either as a crude extract or as purified compounds.

For example, the leaf surfaces of plants are a rich source of phytochemicals. In addition to their internal components, leaf surfaces of higher plants are covered with non-cellular cuticular materials, which are

non-living, and heterogeneous in chemical composition such as lipids, wax and cutin (biopolymer composing fatty and hydroxy fatty acids), as well as proteins and many secondary metabolites associated with leaf surface or present in hairs or trichomes covering leaf surfaces. These cuticular compounds can be assessed or removed by rapid immersion of intact leaves in organic solvents or by running the solvents over the leaf surface. It is generally agreed that these techniques avoid contamination by substances within the leaf making the process significantly different from total tissue extraction. The amounts of cuticular compounds present on the leaf surfaces of different species are variable, but normally lie in the range 0.01-0.5mg/cm^2. More cuticular compounds are usually obtained from the lower than the upper leaf surface. Waxes and other cuticular compounds are deposited in early stage of leaf growth and continues throughout the period of leaf expansion. Leaf waxes are known to inhibit spore germination of pathogenic fungi.

In addition to leaves, plant roots continuously produce and secrete a characteristically unique set of compounds into their immediate environment (rhizosphere). While up to 10% of photosynthetically fixed carbon is secreted from the roots in the form of biologically active compounds, the systematic study of compounds present in root exudates of diverse plant species and their biological activity has not been undertaken. However, certain compounds present in root exudates have been shown to play an important role in several biological processes, including activation of the *Rhizobium* genes responsible for the nodulation process and, possibly, for vesicular-arbuscular mycorrhiza (VAM)

colonization. Strigol, a germination stimulant for the parasitic plant *Striga asitica*, has been found in the root exudates of many cereals. In addition, root-secreted compounds called phytosiderophores may be involved in the acquisition of essential plant nutrients from soils and in defense against such toxic metals as aluminum. While many biologically active compounds were isolated from extracts of plant roots, screening the root exudates for compounds and isolation of biologically active or anti-microbial compounds from exudates have not been investigated.

In general, compounds have been recovered from roots and leaves and used either as a crude root exudate extract or as purified compounds which require the use of complex extraction and purification procedures. Heretofore, it was not known that one could identify and recover collections (libraries) novel agents which have biologic activity which are exuded from a plant part, e.g., onto the surface of the leaf or in the media in which roots are contained.

SUMMARY OF THE INVENTION

This invention provides a method of identifying and recovering chemical compounds or other substances exuded from a plant or plant part. In a preferred embodiment, the invention is directed to recovering compounds from plants which have been subjected to treatments or conditions to induce and increase production of such compounds in the plant. The compounds recovered and identified preferably possess one or more biological activities, including but not limited to such activities as antibacterial, antifungal, anti-herbicidal, insecticidal, anti-cancer, sporicidal cytotoxic

activities, smell, taste, scents, and flavor enhancement.

According to one aspect of the invention, a method is provided for recovering and identifying biologically active compounds exuded from a portion of a plant covered with a cuticle (e.g., a leaf surface), the method comprising: (a) removing cuticular material located on the surface of the portion of the plant by contacting the leaf surface with a solvent capable of partially or completely dissolving the cuticular material; (b) assaying the solvent containing the cuticular material for the identification of agents of the cuticular material which have biological activity; and (c) analyzing the solvent containing the cuticular material so as to identify the agent or agents which have the biological activity.

In one embodiment the biological activity is anti-microbial, herbicidal, or one which provides smell, taste, or flavor enhancement. Examples of anti-microbial activity includes antibacterial or antifungal activity. In another embodiment, the step of assaying the solvent solution comprises contacting the solution or its components with a medium containing a suspension of a microorganism, wherein the inhibition of the growth of the suspension of the microorganism is indicative of an agent in the solvent solution having biological activity. The medium may be a liquid medium or an agar medium.

In another embodiment the cuticular material is a lipid, wax, cutin, protein, primary or secondary metabolite. In another embodiment the solvent is an organic solvent. Examples of solvents include, but are not limited to, methylene chloride and chloroform. In another embodiment the suspension of microorganism is a bacterium, fungus, or virus. Examples of microorganism

include but are not limited to the following: *Escherichia coli* K-12. F, prototropic Str., *Staphylococcus aureus* subsp. *Aureus*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Aspergillus flavus* and *Penicillium nigra*.

In another embodiment the plant is selected from a group consisting of: *Atropa belladonna*, *Erythrinia glabeliferus*, *Ipomea tricolor*, *Erythrinia crista*, *Celosia cristata*, *Gallium sporium*, *Laurus nobilis*, *Vitis labrissa*, *Gratiola officinalis*, *Symphitium officinalis*, *Hosta fortuna*, *Casia hebecarpa*, *Thalictum flavum*, *Scutellarian altissima*, *Portulaca oleacea*, *Scutellaria certicola*, *Physalis creticola*, *Geum fanieri*, *Gentiana tibetica*, *Linum hirsutum*, *Aconitum napellus*, *Podophyllum amodii*, *Thymus cretaceus*, *Hosta fortunaea*, *Carlina acaulis*, *Chamaechrista fasciculata*, *Pinus pinea*, *Pegamun hamalis*, *Tamarindus india*, *Carica papaya*, *Cistus incanus*, *Capparis spinosa inemis*, *Cupress lusitanica*, *Diopiros kaka*, *Erungiurn campestre*, *Aesculus woerlitzenis*, *Aesculus hippocastanum*, *Cupressus sempervirens* and *Celtis occidentalis*.

In another embodiment, the step of analyzing the cuticle-containing solvent furthers comprises fractioning the agents identified in the solvent. In another embodiment, subsequent to fractioning the solution, the resulting agent is identified.

This invention also provides a method of recovering and identifying agents, preferably biologically active compounds, exuded from or onto the roots of a plant, the method comprising: (a) obtaining a sample of a medium (e.g., an aqueous medium such as water buffer or liquid growth medium) which contacted plant roots; (b) assaying the sample for biological activity; and (c) analyzing the sample so as to identify the agents

which have the biological activity. This method preferably is performed on a living plant, the roots of which are maintained in contact with the medium in order to recover from the medium one or more compounds produced by the plant.

In one embodiment, the method comprises adding an elicitor to the aqueous media prior to removal of the roots from the media. In another embodiment the method further comprises adding an elicitor to the aqueous media during the growth of the roots. In another embodiment the elicitor is an abiotic or biotic elicitor. Examples of biotic elicitors include but are not limited to the following: chitosan, or fungal and bacterial cell wall fragments, methyl salicylate and methyl jasmonate. Examples of abiotic elicitors include, but are not limited to, silver nitrate and acetic acid.

In another embodiment, the plant is subjected to conditions known to induce production of novel compounds, or to increase production of compounds of interest, after which such compounds are recovered from the medium contacting the roots. Such conditions include heat stress, drought stress, salt stress, over- or under-illumination and nutrient deprivation, among others.

In accordance with a further aspect of the invention, there is generated a chemical compound library which may be used for screening for a desired compound or activity. In one embodiment, plants or plant parts which are specifically grown or maintained for the purpose of recovering compounds therefrom are contacted with water or another aqueous medium while alive in order to recover a variety of compounds for potential screening. In another embodiment, cuticle covered plant parts are treated with a cuticle-dissolving solvent in order to

recover compounds from cuticular material. The plants or plant parts are preferably contacted with an elicitor, or subjected to the growth conditions summarized above, to increase the amount and/or diversity of compounds which can be recovered in the plant exudates.

Other features and advantages of the present invention will be appreciated from the drawings, detailed description of the invention, and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Agar plate showing antimicrobial activity of the root exudate of *Laurus nobilis (1881)* against *Escherichia coli*.

Figure 2. Agar plate showing antimicrobial activity of the root exudate of *Gentiana tibetica (1881)* against *Escherichia coli*.

Figure 3. Agar plate showing antimicrobial of the root exudate of *Aconitum napellus* (1881) against *Escherichia coli*.

Figure 4. Agar plate showing antimicrobial activity of the leaf surface compounds (identified on the Figure as samples) of *Erythrina christagalli* (1363) against *Staphylococcus aureus*.

Figure 5. Agar plate showing antimicrobial activity of the leaf surface compounds (identified on the Figure as samples) of *Laurus nobilis* (1513) against *Staphylococcus aureus*.

Figure 6. Agar plate showing antimicrobial activity of the root exudate of *Scutellaria altissima* (1671) against *Staphylococcus aureus*.

Figure 7. Agar plate showing antimicrobial activity of the root exudate of *Scuttellaria creticola* (1691) against *Staphylococcus aureus*.

Figure 8. Agar plate showing antimicrobial activity of the root exudate of *Hosta fortuna* (1645) against *Saccharomyces cerevisiae*.

Figure 9. Agar plate showing antimicrobial activity of the root exudate of *Cunninghamia lancelota* (2489) against *Aspergillus flavus*.

Figure 10. Agar plate showing antimicrobial activity of leaf surface compounds (identified on the Figure as samples) from leaves of *Thymus citriodorus* "aureus" (59) and Hydrocotyle asiatica (32a) against *Staphylococcus aureus*.

Figure 11. Agar plate showing antimicrobial activity of leaf surface compounds (identified on the Figure as samples) from leaves of *Betula pendula* (24) against *Staphylococcus aureus*.

Figure 12. Agar plate showing antimicrobial activity of leaf surface compounds of *Eucalyptus radus* (229) against *Staphylococcus aureus*.

Figure 13. Agar plate showing antimicrobial activity of leaf surface compounds of *Eucalyptus radus* (229)against *Saccharomyces cerevisiae*.

Figure 14. Agar plate showing antimicrobial activity of leaf surface compounds of *Oreopanax capitata* (216) against *Staphylococcus aureus*.

Figure 15. Agar plate showing antimicrobial activity of leaf surface compounds of *Oreopanax capitata* (216) against *Escherichia coli*.

Figure 16. Graphical presentation of the amount of daidzein recovered from root exudates produced by soybean plants treated with different elicitors.

Figure 17. Graphical presentation of the amount of genistein recovered from root exudates produced by soybean plants treated with different elicitors.

Figure 18. HPLC profile of the diversity of compounds recovered from root exudates of various plants.

Figure 19. HPLC profile of the diversity of compounds recovered from *Lupinus luteus*.

Figure 20. HPLC profile of the diversity of compounds recovered from root exudates of *Brassica juncea*.

Figure 21. HPLC profile of the diversity of compounds recovered from root exudates of *Datura metel*.

Figure 22. HPLC profile of the diversity of compounds recovered from root exudates of *Lupinus polyphyllus*.

Figure 23. HPLC profile of the diversity of compounds recovered from root exudates of *Meliltotus medicaginoides*.

DETAILED DESCRIPTION OF THE INVENTION

The plant or plant portion that is subjected to one or more of the processes of the present invention is a living plant, or obtained from a living plant. The plant may be any plant as described in greater detail herein. Any portion of the plant may be utilized, including but not limited to, leaves, shoots, seeds, seedlings, stems, flowers and roots.

As defined herein "cuticular material" means cutin and other materials located in the cuticle covering the leaf surface or leaf hairs or trichomes of a plant.

As defined herein "exudate" refers to substances which are exuded, secreted, released or deposited from a plant or plant part, either onto the surface of the plant or plant part, or into the surrounding medium.

As defined herein, "antimicrobials" means the

spectrum of organisms against which they are active, whether they kill the organism or merely slow its growth and reproduction (i.e., cidal or static) and the biochemical system on which they exert their major biochemical action (e.g., inhibit protein synthesis or cell wall synthesis). Antimicrobials include agents acting against any microorganism, including gram positive or gram negative bacteria, Rickettsia, fungi or protozoans.

The plant preferably is a higher plant. It is contemplated by this invention that any plant may be employed in the method. For example, the following plants may be employed in order to identify agents which are exuded: *Atropa Belladonna, Erythrinia glabeliferus, Ipomea tricolor, Erythrinia crista, Celosia cristata, Gallium sporium, Laurus nobilis, Vitis labrissa, Gratiola officinalis, Symphitium officinalis, Hosta fortuna, Casia hebecarpa, Thalictum flavum, Scutellarian altissima, Portulaca oleacea, Scutellaria certicola, Physalis creticola, Geum fanieri, Gentiana tibetica, Linium hirsutum, Aconitum napellus, Podophyllum amodii, Thymus cretaceus, Hosta fortunaea, Carlina acaulis, Charnaechrista fasciculata, Pinus pinea, Pegamun hamalis, Tamarindus india, Carica papaya, Cistus incanus, Capparis spinosa inemis, Cupress lusitanica, Diopiros,kaka, Erungium campestre, Aesculus woerlitzenis, Aesculus hippocastanum, Cupressus sempervirens* and *Celtis occidentalis.*

Further plants species used for screening exudates are as follows: *Polygonum cuspidatum, Eleagnus angustifolia, Eleagnus cemutata, Gentiana macrophilla, Brassica napa, Sesbania exaltata, Sesbania speciosa, Spartina potentiflora, Brassica juncea, Helianthus annus,*

Puansetia sp., Pelargoniurn zonale, Sundapsis spp., Leontopodium alpinum, Lupinus luteaus, Buxus microphilla "japonica", Liatris spinata, Rimula japonica, Betula nigra, Filipendula vulgrais, Lobelia siphitica, Gravilia robusta, Reseda luteola, Gentiana littorala, Campanula carpatica, Aesculus hypocastanum, Aesculus waertilensis, Ageratum conizoides, Psidium guajava, Ailantus altissima, Buxus microphylla "japonica", Hydrocotile asiatica, Gravilea robusta, Brugmansia suaveolens, Thymus puliglodes, Thymus lemabarona, Thymus serphylum (wild), Gaultheria procumbens, Thymus serphylum, Thymus camosus, Thymus thrasicus, Calicatus floridus, Zingiber officinalis, Lapia dulcis, Thymus vulgaris "argenteus", Thumus praecox "arcticus", Thymus puleglodes "lemons", Thymus speciosa, Thymus carnosus, Thymus pseudolamginosus, Thymus praecox, Thymus vulgaris "oregano", Ficus religiosa, Forsithsia suspensa, Chelidonium majus, Thymus wooly, Thymus portugalense, Nicotiana tabacum, Thymus cytridorus "aureus", Thymus vulgaris, Cactus officinailis, Lal lab purpurea, Juglands regia, Actinidia chinensis, Hernerocalis spp., Betula pendula, Gardenia jasminoides, Taxodium dixticum, Magnolia loebheril, Crataegus praegophyrum, Larix dedidua, Tuja orientalis "eligantissima", Tula ocidentalis "columbia", Xeupressocyparis deylandii, Pseudotsuga menzisia, Abies firma, Fautenousus qualiqualia, Alium cernum (wild), Juniperus "blue pacific", Taraxacum officinalis, Juca sp., Ilex agnifolium, Tsuga canadensis "penola", Tsuga canadensis "penola", Ilex cornuta, Taxus xiksii, Taxus media, Metasequoia glyptotrobioldes, Pinus bungiana, Boxus sempervirens, Stevartia coreana, Prunus xocane, Betula daurica, Plantago minor, Acer palmaturn "burgundy", Acer campestre, Cotynus cogygria, Quercus robur "fastigiata",

Acer truncatum, Archirantus bidentata, Alum japonica, Carum capsicum, Agastache mexuicana, Prunella vulgaris, Tagetes minuta, Nepeta cataria, Ratibiunda columnus-Fera, Aster-Nova anglicae, Mirica certifera, Pittisporum tibica, Taxodium dixticum (H_20), Taxodium dixticum (Acetic acid), Plantago major, Scotch pine, Asorum canadiensis, Pieras japonica, Pinus sirtrobus, Trifolium pratense, Prunus serotica, Darura stramonium, Geranium maculata, Hydrocotile asiatica, Astragulus sinicus, Centauria maculata, Ruschia indurata, Myrthus comunis, Platanus acidentalis, Liclum barbatum, Lavandula officinalis, Gravilea robusta, Hyppoach rhamnoides, Filipendula ulmaria, Betula pendula Polygonium odoratum, Brugmansi graveolens (ralf), Rhus toxicodenta, Armoraica ristica, Ficus benjaminii, Sluffera sp., Pelagonium zonale, Allium sp. Asimina triloba, Lippa dulcis, Epilobium augustifolium, Brugmansia suavecolens (old), Brugmansia suaveolens (young), Xanthosoma sagittifolium (leaf), Xanthosoma sagittifolium (stem), Monstera deliciosa, Aglaonema commutatus, Dieffenbachia leopoldii, Anthurium andreanum, Syngoniurn podophyllum, Dracaena fragrans, Ananas comosus, Strelitzia reglinae, Diffenbachia segiunae, Syngoniurn aurutum, Dracaena sp., Hhaemanthus katharina, Anthurium altersianum, Spathiphyllum grandiflorum, Spathiphyllum cochlearispaturn, Monstera pertusa, Anthurium magnificurn, Anthurium hookeri, Anthurium elegans, Calathea zebrina, Yucca elephantipes, Bromelia balansae, Musa textilis (Leaf), textilis (Stem), Myrthus communis, Olea olcaster, Olea europaea, Verium oleander, Cocculus laurifolius, Microsorium punctatum, Ficus sp., Senseviera sp., Adansonia digitata, Boechimeria boloba, Piper nigrum, Phymatosorus scolopendria, Turnera ulmifolia, Nicodemia diversifolia, Tapeinochilos spectabilis, Rauwolfia tetraphylla, Ficus

elastica, Cycas cirinalis, Caryota ureus, Cynnamonum zeylonicum, Aechmea luddemoniana, Foenix zeulonica, Ficus benjamina, Ficus purnila, Murraya exotica, Trevesia sungaica, Clerodendrurn speciossicum, Actinidi colonicta, Paeonia lactiflora, Paeonia suffructicisa, Quercus imbricaria, Iris alida, Portulaca olleracea, Poligonum aviculare, Iris pseudocarpus, Allium nutans, Allium fistulosum, Antericum ramosum, Veratrum nigrum, Poligornun latifolia, Hosta lancefolia, Hosta zibalda, Echinops sphae, Paeonia daurica, Inula hilenium, Trambe pontica, Digitalis lutea, Bactisia australis, Austolachia australis, Hissopus zerauchariсus, Feucrium hamedris, Sedum album, Heraclelum pubescens, Origanurn vulgare, Cachris alpina, Haser trilobum, Matteucia strutioptoris, Sedum telchium, Bocconia cordata, Hiuga reptans, Talictrum minus, Anemona japonica, Clematis rectae, Talictrum sp., Alchemilla sp., Potentilla alba, Poterium sangiusorba, Minispermum dauricum, Oxobachus nictogenea, Armoracea rusticana, Cramble cardifolia, Agrimonia eupatora, Uschusa sp., Polymonium ceruleum, Valeriana offocionalis, Pulmonaria molissima, Stachis lanata, Coronolla varia, Platicada grandiflora, Lavandula officinalis, Vincetocsicum officinalis, Acolypha hispida, Gnetum guemon, Psychotria nigropunctata, Psychotria metbacteriodomasica, Cobiaeum varilaturn, Phyllanthus grandifolium, Pterigota alata, Pachyra affinis, Sterulia elata, Phylidendron speciosus, Pithecelobium unguis, Sanchezia nobilis, Oreopanax capitata, Ficus triangularis, Pigelia pennata, Piper chaba, Laurus nobilis, Erythrinia caffra, Metrosideros excelsa, Osmanthus spp., Cupressus sempervirens, Jacobinia sp., Senecio platifilla, Livistona fragrans, Tetraclinis articulata hinensis, Eucaliptus rudis, Podocarpus spinulosus, Eriobotria japonica, Gingko biloba,

Rhododendron spp., Thuja occidentalis, Fagopyrum suffruticosum, Geum macrophullum., Magnolia cobus, Vinca minor, Convalaria majalis, Corylus avelana, Barbaric sp., Rosa multiflora, Ostrea carpinifolia, Ostrea connote, Quercus rubra, Tulip tree, Sorbus aucuparia, Betula nigra (leaf), Betula nigra (flower), Castanea sativa Bergenia crassifolia, Artemisia dracunculus, Ruta graveolens, Quercus nigra, Schisandra chinensis, Betula alba, Sambucus niora, Gentiana cruciata, Encephalaris horridum, Phebodium aureum, Microlepia platphylla, Ceratoramia mexicana, Stepochlaena tenuifolia, Adianthum trapezieformis, Adianthum radiatum, Lycodium japonicum, Aessopteria crasifolia, Asplenium australasicum, Agatis robusta, Osmunda regalis, Osmundastrum claytonionum, Phyllitis scolopendrium, Polyschium braunii, Crytomium fortunei, Dryopteris filis-max, Equisetum variegatum, Anthyrium nopponicum, Anthyrium filis-femina, Parthenosicus tricuspidata, Ligustum vulgare, Charnaeciparis pisifera, Rosa cocanica, Citinis coggriaria, Pinus strobus, Celtis occidentalis, Picea schrenkiana, Cydonia oblonga, Ulmus pumila, Euonomus verrucosa, Deutria scabra, Mespilus germanica, Quercus castanufolia, Euonomus europea, Seruginea suffruticisa, Keyleiteria paniculata, Seringa josiceae, Zelcova, carpinifolia, Abies cephalonica, Taccus bacata, Taxus cuspidata, Salis babilonics, Thuja occidentalis, Actinidia colomicta, Magonia agrifolia, Aralis mandshurica, Luglands nigra, Euonimus elata, Princepia sp., Forsitsia europea, Sorbocotoneaster sp., Morus alba, Crategus macrophyllum, Eucomia ulurifolia, Sorbus cominicta, Philodendron amurense, Comus mass, Korria japonica, Parrotia persica, Jasminum frutocarus, Sulda sanganea, Pentaphylloides fruticosa, Sibirea altaiensis, Cerasus japonica, Kolkwitzia amabilis, Amigdalus nana,

Acer mandshurica, Salix tamarisifolia, Amelanchier spicata, Cerasus maghabab, Prunus cerasifera, Coryllus avelana, Acer tataricum, Viburnum opulus, Siringa vulgaris, Fraxinus exelsior, Quercus trojana, Chaernomelis superba, Pinus salinifolia, Berberis vulgaris, Cotoneaster horisontalis, Cotoneaster fangianus, Fagus silvatica, Pinus pumila, Pinus silvestris and *Berberis thungergi.*

Recovery of substances exuded into aqueous media:

In one aspect of the invention, compounds exuded from the plant into a surrounding aqueous medium are recovered and thereafter identified or otherwise analyzed. The aqueous medium may be water, which may or may not contain other constituents, such as nutrients, elicitors and similar substances commonly found in hydroponic plant culture media.

In accordance with a preferred aspect, the chemical compounds are those which are exuded, secreted or leached from the plant, preferably from the root or plant seedling or shoot. In the case where seedlings or shoots are used, the plant seed, shoot or root may be treated with an elicitor or inducer to increase production in the plant of one or more produces, as described in greater detail below. The inducer or elicitor may be in the aqueous solution that contacts the plant for recovery of the exuded compounds, or may be separately applied to the plant.

The contact with the aqueous medium may be effected by placing at least the plant roots in water, or by "aeroponics", which involves contacting the plant, particularly the roots, with water droplets from which chemical compounds are recovered.

In a preferred embodiment, secreted substances are recovered from a plant in a continuous process by having the plant roots (or rooted shoot) of the plant or shoot in contact with the aqueous medium, which may be continuously or periodically passed over the roots to recover the substances secreted into the medium. The aqueous medium is then assayed for biological activity, or further subjected to processes for recovering the substances contained therein.

In another embodiment, seedlings are employed as a source of the phytochemicals. In this embodiment, seeds are germinated in aerated aqueous medium, which is recovered on a continuous or semi-continuous basis in order to obtain and identify the secreted substances.

Thus, a preferred embodiment of the invention provides a method of identifying an agent from root exudates of a plant having biological activity, the method comprising: (a) obtaining a sample of media which contacted plant roots; (b) assaying the sample for biological activity; and (c) analyzing the sample so as to identify the agent which has the biological activity.

In any of the embodiments set forth above, the method may further comprise treatment with an elicitor or inducer. The elicitor may be an abiotic or biotic elicitor. Examples of biotic elicitors include but are not limited to the following: chitosan, fungal or bacterial cell walls or fragments thereof, cultivation medium, methyl salicylate and methyl jasmonate. Examples of abiotic elicitors include but are not limited to silver nitrate, or acetic acid. The plants, in particular roots thereof, may be contacted with an elicitor or inducer, which is a chemical compound, for example, organic and inorganic acids, fatty acids,

glycerides, phospholipids, glycolipids, organic solvents, amino acids and peptides, monosaccharides, oligosaccharides, polysaccharides and lipopolysaccharides, phenolics, alkaloids, terpenes and terpenoids, antibiotics, detergents, polvamines, peroxides, ionophores. etc., or subjected to a physical treatment, such as ultra-violet radiation, low and high temperature stress, osmotic stress induced by salt or sugars, nutritional stress defined as depriving the plant of essential nutrients (e.g., N, P or K), in order to induce or elicit increased production of one or more chemicals. Such chemical compound or physical treatment may be applied continuously or intermittently to the plant or plant part. In one embodiment, such treatment may be accomplished by contacting the plant roots with a solution containing the elicitor or by irradiating the plant as temperature stresses. However, the invention is not limited to such an embodiment in that other portions of a plant or seedlings may be contacted with an elicitor. For example, a glycopeptide elicitor may be prepared from germ tubes of the rust fungus *Puccinia graminis* Pers. *f. sp. tritici* Erikss. & Henn (Pgt), as well as chitin oligosaccharides, chitosan, and methyl jasmonate (MJ) stimulated lipoxygenase (LOX) activity (E. C. 1. B. 11. 12) in wheat (*Triticum aestivum*) leaves. The effects of elicitor concentration and exposure time on growth and levels of biologically active compounds vary. For example, transient studies at the same level demonstrated possible catabolism as serpentine, tabersonine, and lochnericine levels decreased immediately after elicitation. The levels of these compounds recovered back to control levels or were higher than the control levels after some time. Jasmonic acid

was found to be a unique elicitor leading to an enhancement in flux to several branches in the indole alkaloid pathway. Jasmonic acid addition caused an increase in the specific yields of ajmalicine 80%), serpentine (60%), lochnericine (15 0%), and horhammericine (500%) in dosage studies. Tabersonine, the likely precursor of lochnericine and horhammericine, decreased at lower levels of jasmonic acid and then increased with increasing jasmonic acid concentration. Transient studies showed that lochnericine and tabersonine levels go through a maximum, then decrease back to control levels and reduce below control levels, respectively. The yields of ajmalicine, serpentine, and horharnmericine increased continuously after the addition of jasmonic acid.

Recovery of compounds from cuticular material:

In another aspect of the invention, compounds exuded from the plant onto or into the cuticle of the plant are recovered by contacting the plant part containing the cuticular material with a solvent that partially or completely dissolves the cuticular material, thereby removing that material from the plant for subsequent identification of compounds of interest disposed therein.

The cuticular material may comprise lipid, wax, cutin, protein, primary or secondary metabolite. Solvents that dissolve such materials generally are composed at least in part of organic solvents. Examples of suitable solvents include, but are not limited to, methylene chloride and chloroform.

Elicitors and other treatments to induce production of novel compounds, or to increase production

of a compound of interest, may be used in this aspect of the invention in a manner similar to their use described above. The plant is treated as described above, prior to contacting the plant part with the cuticle-removing solvent.

Identification and analysis of compounds of interest in plant exudates:

The results presented herein demonstrate that agents having biological activity can be identified by employing the cuticular exudate and root exudate assay. Specifically, a total of 400 plant species cuticular washings has been prepared and tested against microbial/fungal cultures. A significant percentage of cuticular washings shows antimicrobial activity. Figures 4, 5, 10, 11, 12, 13, 14 and 15 demonstrate the results of this activity.

In one embodiment, the step of assaying the various solutions containing the exudates comprises contacting the solution or its components with a medium containing a suspension of a microorganism, wherein the inhibition of the growth of the suspension of the microorganism is indicative of an agent in the exudate having biological activity. The medium may be a liquid media or an agar media. Microorganisms including but not limited to bacteria or fungi, may grow within, or develop in or on the medium. Inhibition of the growth is detected by standard means known to those skilled in the art. For example, the growth inhibition on agar may be measured in terms of zone of inhibition which is known to those skilled in the art.

Two common applications of agar diffusion assays are potency testing of new production lots in the

pharmaceutical industry and bacterial susceptibility testing. They are based on the same principles, but susceptibility assays use unknown bacterial strains, and potency assays use bacterial strains with well characterized performance against the test drug. Agar diffusion potency assays are relatively comparable in sensitivity and accuracy to radiometric enzyme, fluorescent (FIA) &, ELISA. For example, single-plate assay is based on having all standard and unknown concentrations on one single plate. This eliminates plate to plate variation, and facilitates layout and reading. Alternatively, 2 or 3 identical plates may be laid out, examined and averaged. Usually NUNC (Denmark) large 243 mm square plates are used, which allow up to 64 samples that easily fit on one plate. It thus provides for up to 6 standards. and up to 10 samples, with 4 replicates of each standard and unknown sample concentrations, on one plate. Multiple-Plate format uses many 90-100 mm petri dishes, and conforms strictly to US-FDA, US-CFR and US-USP published methodology. Zone diameters are measured by Video or Caliper directly into the software.

The suspension of microorganism may comprise a gram positive or a gram negative bacterium, protozoan, fungus, or virus. Examples of gram positive and gram negative bacteria are known to those skilled in the art. Microorganism include but are not limited to the following: *Escherichia coli K-12. F, prototropic Str., Staphylococcus aureus subsp. Aureus, Pseudomonas aeruginosa, Saccharomyces Cerevisiae, Aspergillus flavus* and *Penicillium nigra*.

In another embodiment, the step of analyzing the solution furthers comprises fractioning the solvent

solution directly or following drying and resuspension step. In another embodiment, subsequent to fractioning the solution, the resulting agent is identified. Fractionation methods are known to those skilled in the art. For example, chromatographic methods, for example HPLC, may be employed to identify the compound. The chromatography separation of extracted products may be employed with an HPLC-system consisting of Waters 996 Photodiode Array Detector (PDA) with usable UV range from 190 to 800 nm; a Waters 717 plus autosampler; two Beckman110B solvent Delivery Modules, connected with a Beckman System Organizer (mixer) and a Beckman System Gold Analog Interface Module 406. The Beckman solvent delivery system is controlled by a NEC PC-8300 computer. Chromatography and spectral data is managed by Waters Millennium chromatography software, version 2.10, using a NEC Image 466es computer. All hardware components, except the solvent delivery system, are connected through a standard IEEE communication system. Compounds are separated 5on a Waters Nova Pak6 C-18 reverse 25 phase column, 3.9 x 150 mm, 60A pore size, and 4μm particle size. Prior to use, each batch of solvent A is digested under vacuum and ultrasonications for 5 minutes. The mobile phase flow is adjusted. to 1ml/min, and a gradient mode of separation is used for all separations. Compounds are detected with PDA detector or with Waters Thermabeam™ Mass Detector.

Further, one may fractionate the sample by chromatography techniques followed by the chemical structure analysis using mass spectroscopy; infra red spectroscopy; or 1D or 2D nuclear magnetic resonance spectroscopy (proton or ^{13}C. Fractionation and analysis methods are known to those skilled in the art. As

demonstrated herein, cuticular washings from *Betula pendula* showed very high activity. After 10 repeat experiments the sample was fractionated by HPLC. From 35 fractions, fraction No. 3 showed very high antibacterial activity for *Staphylococcus aureus*. After analyzing this fraction on LC-MS, a fragmentation pattern of peak A66(the main component in all active fractions) with many steroids as cholestane derivatives and lanostane derivatives resulted.

The methods described in this invention could generally be used in devising strategies for enhancement in productivity of secondary metabolites and for probing and studying the complex secondary metabolite pathways in plant tissue cultures. The methods are considered to be of great utility in recovering novel compounds for use in medicine, agriculture and the food industry, among others.

The following examples are provided to describe the invention in greater detail. They are intended to illustrate, not to limit, the invention.

EXAMPLE 1
Bioassay of Leaf and Root Exudates

Methods

Preparation of cuticular washings: Leaves from plants were contacted with 5 ml of solvent (methylene chloride) contained in a plastic sandwich bags (quart size, 7in x 8in)as containers. To standardize the cuticular wash concentration by relating it to the used leaf surface from which it was taken, approximately 60 cm2 of leaf surface were used. Sandwich bags with zippers were used to guarantee that the surface of leaf

is totally moisturized with solvent. To facilitate the removal of cuticular compounds the bag containing a leaf and the solvent was shaken for approximately 20-40 seconds. The end of bag was cut and the content removed into 20ml scintillation vials,and closed with Teflon or foil faced liner screw caps and stored in refrigerator. Alternatively, the solvent containing the cuticular washings can be dried inside the scintillation vial before cold storage.

Preparation of bacteria and fungus suspensions: 6 different organisms were used for antibacterial and antifungal screening: 1. *Escherichia coli* K-12. F, prototropic Str.; 2. *Staphylococcus aureus* subsp. *Aureus*; 3. *Pseudomonas aeruginosa*; 4. *Saccharomyces cerevisiae;* 5. *Aspergillus flavus*; 6. *Penicillium nigra*. Bacteria (*Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa*) were maintained on solid agar media (LB Agar, Miller). Before screening, bacteria was transferred into liquid media and cultivated for 12 hours at +3 7°C on shaker with a cell density 10^{5-6}. Saccharomyces cerevisiae (yeast), *Aspergillus flavus* and *Penicillium nigra* were cultivated on Potato dextrose media. Before treatment yeast cells were transferred into liquid media and cultivated for 48 hours at +30°C on a shaker. The spores of *Aspergillus flavus* were washed with distilled water from fungus surface grown in Petri dish and resuspended in fresh distilled water.

The standard method used to determine *in vitro* antibacterial and antifungal activity of leaf surface cuticular washings consisted of testing suspension of microorganisms and spores of fungus for growth inhibition in the presence of washings. The antibacterial and antifungal activity was indicated by 30% or more, growth reduction of cells/spores in the presence of cuticular

washings. All samples were plated in 3 replicates. One ml of nutrient media was put in each well in 24 well plates. Thereafter, 10 ul of methylene chloride solution containing cuticular washings were placed on agar surface and after the drop dried (2-3 min) 30 ul of microorganism suspension or fungus spores were plated on top of agar and equally distributed throughout the surface. After 24 hours of incubation at +30°C, the plates were examined for the presence/absence of activity. To test antimicrobial/antifungal activity of root exudates, cell suspension was plated and spread on the agar surface into each of 24 well plates. Using a 5ml Eppendorf pipet tip attached to a vacuum line, a hole was made in the center of each well and 20μl of exudate dissolved in water 5mg/300ml) gently poured into the hole. The following elicitors were employed: methyl salicylate, methyl jasmonate, silver nitrate, acetic acid and chitosan.

To harness the vast and largely unexplored diversity of biological natural products exuded by plant roots, an efficient method for collecting root exudates from various plants was developed. This method is based on a modified hydroponic technology which allows maintaining plant roots in water or diluted nutrient solution followed by of compounds exuded from roots.

The seeds of cultivated and wild species obtained from the commercial seed companies or botanical gardens were germinated in a greenhouse inside a 0.9 cm in diameter, 0.cm deep well cut into Grodan rockwool cubes (3.4 cm width x 3.4 cm depth x 3.7 cm height). Rockwool cubes were placed inside standard greenhouse plastic trays (dimensions 52 cm width x 25 cm depth x 7 cm height)and watered with an overhead misting system. Seeds were allowed to germinate for 3-6 days until the roots started to emerge from the bottom of the rockwool cube.

After germination, the cubes containing the seedlings were inserted into the center of a Styrofoam ring with an inside diameter 3.2 cm, outside diameter 8.2 cm and 2.5 cm thickness. The ring was floated on the surface of 400-800 mL of hydrophic nutrient solution (2 g/L Hydro-Sol [Scotts-Sierra Horticultureal products Comp., Marysville, OH.] supplemented with 1.2 g/L $Ca[NO_3]_2$) contained inside a light impermeable, high density polyethylene cylinder (9.0 cm in diameter, 16 cm in height).

Aeration was provided by shaking the cylinders at 50 rpm on a platform shaker (Labline Orbital Shaker, Model 3590). Seedlings were cultivated hydroponically in this system for 3 to 6 weeks with roots growing in a nutrient solution. Thereafter, the root system (average root dry weight 0.1±0.05g) was removed from the nutrient solution and placed inside a 30 mL glass beaker, containing 10-20 mL of distilled water or distilled water supplemented with an elicitor. To prevent water loss from the plant canopy and drying of the collecting solution, plant shoots were covered with transparent plastic bags. After 24h, unless noted otherwise, a small sample from the root solution was removed and analyzed for the phytosecreted products. This system of hydroponic plant cultivation and exudates collection is referred to as standard exudate collection system. Root exudates may also be freeze-dried and stored in the freezer at -20°C. When needed, the exudate powder may be re-dissolved in water and used for screening or chemical analysis.

Results

A total of 844 plant species root exudates elicited have been prepared and tested against six-above mentioned microbial/fungal cultures (Table II). The

final concentration of exudates used in assay was 5 mg of dry exudate diluted in 300 μl of distilled water. It has been found that great majority of material tested at this concentration did not affect adversely growth of the tested organisms. Figures 1, 2, 3, 6, 7, 8, 9 demonstrate the results of the inhibition of the biological activity. A number of primary hits has been identified against all, but one (Aspergillus) microorganisms tested. The hit rate under the conditions used varied between 0% and 7.8% (Table 1). It is noteworthy that majority of the hits come from exudates from elicited roots. The unusually high proportion of hits in the materials elicited by silver may also be partially explained by the toxic effects of silver on a given microorganism.

Table 1. Frequency of antimicrobial effects on root exudates

Target Organism	**Number of hits**	**Hit Rate (%)**
Escherichia coli	23 (884)*	2.6
Staphylococcus aureus	34(884)	3.8
Pseudomonas aeruginosa	8(102)	7.8
Aspergillus flavus	0(510)	0
Penicillium nigra	4(102)	3.9
Saccharomyces cerevisiae	6(718)	0.8

* Number in parenthesis indicate total number of root exudate samples tested for a particular microorganism.

Table II

List of plant species from which root exudates show antimicrobial/antifungal activity. The strengths of activity is denoted by the number of "*", with a larger number of "*" referring to a greater activity.

Af - Aspergillus flavus
Ec - Escherichia coli
Sa - Staphylococcus aureus
Sc - Saccharomyces cerevisiae
Pn - Penicillium nigra
Pa - Pseudomonas aeruginosa

	#	Plant Name	Elicitor	Af	Ec	Sa	Sc	Pn	Pa
1	845	Atropa belladonna	Silver			*			
2	857	Erythrina glabelliformis	Silver			*			
3	949	Ipomea tricolor	Silver			*			
4	1363	Erythrina galli	Silver			*			
5	1475	Celosia cristata	Acetic a.		*	*			
6	1501	Gallium spurium	Acetic a.			*			
7	1513	Laurus nobilis	Silver		*	*			
8	1563	Vitis labrusca	Control		*				
9	1585	Gratiola officinalis	Control			*			
10	1617	Symphytum officinalis	Silver		*				
11	1645	Hosta fortunea	Acetic a.				*		
12	1649	Cassia hebecarpa	Control			*			
13	1659	Thalictrum flavum	Acetic a.		*	*			

14	1671	Scutellaria altissima	Silver		*	*			
15	1681	Portulaca oleracea	Silver				*		
16	1683	Portulaca oleracea	Chitosan				*		
17	1685	Portulaca oleracea	Meta				*		
18	1691	Scutellaria creticola	Silver			*			
19	1695	Physalis ixocarpa	Silver			*			
20	1757	Geum fourieri	Control				*		
21	1791	Gentiana tibetica	Silver		*	*			
22	1875	Linum hirsutum	Acetic a.				*		
23	1879	Aconitum napellus	Acetic a.						
24	1881	Aconitum napellus	Silver		*	*			
25	1887	Podophyllum aunodii	Silver						
26	1897	Thymus cretaceus	Silver					*	
27	1913	Hosta fortunea	Silver		***	***		*	
28	1975	Hosta fortunea	Chitosan		**	***			
29	1985	Carlina acaulis	Silver		*	***			
30	2003	Charnaechrista fasciculata	Silver		**	***		*	
31	2013	Pinus pinea	Silver		**	***		**	
32	2043	Peganum harmala	Silver			*			
33	2053	Tamarindus india	Silver		*	***			
34	2063	Carica papaya	Silver		*	***			
35	2111	Cistus incanus	Control		***	**			
36	2161	Capparis inermis	Silver		***	***			***

37	2117	Cupressus lusitanica	Silver		**	***			***
38	2133	Diopiros khaki	Acetic a.			**			
39	2135	Diopiros khaki	Silver		**	***			***
40	2137	Diopiros khaki	Chitosan			**			
41	2145	Eryngium campestre	Silver		**	**			***
42	2255	Aesculus woerlitzienis	Silver		***	***			***
43	2265	Aesculus hippocastanum	Silver		***	***			***
44	2299	Cupressus sempervirens	Silver			***			***
45	2315	Celtis occidentalis	Silver		**	***			***
46	2325	Calycanthus floridus	Silver		**	**			
47	2335	Chimonanthus praecox	Control		**	**			
48	2345	Clematis manschurica	Silver		**	**			
49	2377	Liatris spicata	Silver			*			
50	2379	Liatris spicata	Silver			*			
51	2407	Cladium mariscus	Silver		**	***			
52	2425	Lablab purpureus	Silver			**			
53	2455	Campanula carpatica	Silver			*			
54	2443	Chilopsis linearis	Silver			*			
55	2461	Thuja occidentalis	Silver		**	**			

56	2479	Cosmos sulphureus	Silver		**	**	**		
57	2489	Cunninghamia lanceolata	Silver		**	**	**		
58	2503	Euptelea pleiosperma	Silver			**			
59	2535	Juglands regia	Acetic a.		**	**			

A total of 400 plant species cuticular washings has been prepared and tested against six-above mentioned microbial or fungal cultures and the amount of cuticular washings used for each well was 10 μl. It has been found that a majority of materials tested at this concentration did not affect adversely growth of the tested organisms. However, a significant percentage of cuticular washings showed antimicrobial activity. Figures 4, 5, 10, 11, 12, 13, 14, 15 demonstrate the results of this activity. A number of strong antimicrobial hits have been identified for *Escherichia coli*, *Staphylococcus aureus* and *Saccharomyces cerevisiae* (Table III). The hit rate among cuticular washings from different plant species varies from 0.5 to 5.0% depending on the microorganism.

Table III. List of plants which leaf surface cuticular washings show strong antifungal or antimicrobial activity

	Sample Identification	Plant name	Sa	Ec	Sc
1	125	Taxodium dixticum	X		
2	133	Gravilea robusta	X		
3	136	Betula pendula	x		
4	171	Anthurium elegans	x		
5	198	Foenix zeulinica		X	X
6	216	Oreopanax capitate	X	X	
7	229	Eucaliptus rudis	X	X	X
8	248	Betula nigra	X		
9	274	Paeonia daurica			
10	276	Betula alba	X	X	
11	294	Talictrum sp.	x	X	
12	302	Agrimonia eupatori	X		
13	355	Salix babilonics	X		
14	377	Cerasus janonica			

EXAMPLE 2

Sniffing Test of Root Exudates and Cuticular Washings

The assay was done by thawing a frozen sample to a room temperature, opening a glass vial containing a sample, sniffing it, and immediately marking the results. Samples are stored frozen at -20°C in tightly sealed glass vials (5 ml vials for cuticular washings and 20 ml vials for root exudates). The amount of root exudates in each vial ranges from 5 to 500 mg. The amounts of cuticular compounds in each vial ranges from 5 to 100 mg.

As demonstrated by Table IV and V a significant proportion, 36 out of 100 tested samples of exudates, and 20 out of 100 tested cuticular washings have a strong fragrance. Root exudates were treated with Acetate 03 0.1% acetic acid, AgNO3-20.5 mM $Ag(NO_3)_2$, Chito 02 0.1% chitosan, water, HSL 01 200 μM N-hexanoyl homoerinelactone, and MeJa 03 100 methl jasmonate. In Table IV and V, smell was rated by scientist as follows: 0 no smell; 1 light smell;2 medium smell; 3 strong smell.

Table IV: Sniffing test on root exudates

Sample#	rating	family	genus	species	treatment
1199	3	Solanaceae	Hyoscamus	niger	Acetate 03
1201	3	Solanaceae	Hyoscamus	niger	AgN03 02
1203	2	Solanaceae	Hyoscamus	niger	Chito 02
1205	3	Fabaceae	Genista	tinctoria	Acetate 03
1207	2	Fabaceae	Genista	tinctoria	AgN03 02
1209	2	Fabaceae	Cicer	arietinum	control 01
1211	3	Fabaceae	Cicer	arietinum	Acetate 03
1213	3	Fabaceae	Cicer	arietinum	AgN03 02
1215	3	Fabaceae	Cicer	arietinum	Chito 02
1217	1	Fabaceae	Cicer	arietinum	HSL 01
1219	2	Fabaceae	Thermopsis	fabacea	HSL 01
1221	3	Fabaceae	Thermopsis	fabacea	acetate 03
1223	2	Cucurbitaceae	Trichosanthes	kirilowii	control 01
1225	3	Cucurbitaceae	Trichosanthes	kirilowii	acetate 03
1227	1	Cucurbitaceae	Trichosanthes	kirilowii	HSL 01
1229	2	Cucurbitaceae	Trichosanthes	kirilowii	Chito 02
1233	0	Asteraceae	Xanthium	sibiricum	HSL 01

1235	3	Solanaceae	Brugmansia	suaevolens	AgN03 02
1237	0	Solanaceae	Brugmansia	suaevolens	HSL O1
1239	1	Asteraceae	Eclipta	alba	control 01
1241	3	Asteraceae	Eclipta	alba	Acetate 03
1243	2	Asteraceae	Eclipta	alba	AgN03 02
1245	0	Asteraceae	Eclipta	alba	Chito
1247	3	Asteraceae	Aremisia	absinthium	control 01
1249	3	Asteraceae	Arternsia	absinthium	Acetate 03
1251	2	Asteraceae	Arternsia	absinthium	AgN03 02
1253	0	Asteraceae	Arternsia	absinthium	Chito 02
1255	1	Asteraceae	Silybum	marianum	control 01
1257	2	Asteraceae	Silybum	marianum	Acetate 03
1259	3	Asteraceae	Silybum	marianum	AgN03 02
1261	3	Asteraceae	Silybum	marianum	Chito 02
1263	2	Asteraceae	Silybum	marianum	MeJa 03
1267	3	Apiaceae	Cnidium	monnieri	Acetate 02
1269	2	Apiaceae	Cnidium	monnieri	AgN03 02
1271	2	Apiaceae,	Cnidium	monnieri	Chito 02
1275	2	Apiaceae	Cnidium	monnieri	HSLOI
1277	3	Solanaceae	Brugmansia	suaevolens	control 01
1279	2	Solanaceae	Brugmansia	suaevolens	Acetate 03
1281	1	Clusiaceae	Hypercum	perforatum	control 01
1283	2	Clusiaceae	Hypercum	perforatum	Acetate 03
1285	1	Clusiaceae	Hypercum	perforatum	AcgN03 02
1287	1	Clusiaceae	Hypercum	perforatum	Chito 02
1289	3	Clusiaceae	Hypercum	perforatum	MeJa 03
1291	3	Boraginaceae	Anchusa	officinalis	control 01
1293	2	Boraginaceae	Anchusa	officinalis	Acetate 03
1295	1			sp.	HSL 01

1297	3	Asteraceae	Xanthium	sibiricum	AgNO3 02
1299	3	Zygophilla-ceae	Larrea	tridentata	control 01
1301	3	Zygophylla-ceae	Larrea	tridenta	Acetate 03
1303	2	Zygophylla-ceae	Larrea	tridenta	AgN03 02
1305	0	Zygophylla-ceae	Larrea	tridenta	Chito 02
1307	2	Zygophylla-ceae	Larrea	tridenta	MeJa 03
1309	2	Lamiaceae	Scutellaria	baicalensis	control 01
1311	2	Lamiaceae	Scutellaria	baicalensis	Acetate 03
1313	2	Lamiaceae	Scutellaria	baicalensis	AgN03 02
1315	0	Lamiaceae	Scutellaria	baicalensis	Chito 02
1317	3	Fabaceae	Cytissus	scoparius	control 01
1319	1	Fabaceae	Cytissus	scoparius	Acetate 03
1321	3	Apocynaceae	Rauvolfia	caffra	Chito 02
1333	1	Cyperaceae	Cyperus	esculentus	control 01
1325	3	Cyperaceae	Cyperus	esculentus	Acetate 03
1327	2	Cyperaceae	Cyperus	esculentus	AgN03 02
1329	2	Cyperaceae	Cyperus	esculentus	Chito 02
1331	0	Asteraceae	Arnica	chamissois	HSL OI
1333	3	Solanaceae	Physalis	ixocarpa	control 01
1335	3	Solanaceae	Physalis	ixocarpa	Acetate 03
1337	2	Solanaceae	Physalis	ixocarpa	AgN03 02
1339	0	Solanaceae	Physalis	ixocarpa	Chito 02
1341	1	Solanaceae	Physalis	ixocarpa	MeJa 03

1343	3	Apiaceae	Angelica	polymorh a-sinesis	control 01
1345	2	Apiaceae	Angelica	polymorh a-sinesis	A-NO3 02
1347	3	Apiaceae	Angelica	polymorh a-sinesis	AgN03 02
1349	2	Apiaceae	Angelica	polymorh a-sinesis	Chito 02
1351	1	Apiaceae	Angelica	polymorh a-sinesis	MeJa 03
1353	2	Rosaceae	Agrimonia	pilosa	control 01
1357	3	Asteraceae	Amica	Chamissois	Acetate 03
1359	0	Fabaceae	Erythrina	christa-galli	control 01
1361	3	Fabaceae	Erythrina	christa-galli	Acetate 03
1363	2	Fabaceae	Erythrina	christa-galli	AgNO3 02
1365	1	Fabaceae	Erythrina	christa-galli	Chito 02
1367	0	Fabaceae	Erythrina	christa-galli	HSL OI
1369	3	Ranunculaceae	Aquilegia	vulgaris	control 01
1371	1	Ranunculaceae	Aquilegia	vulgaris	Acetate 03
1373	1	Ranunculaceae	Aquilegia	vulgaris	AgN03 02
1375	1	Ranunculaceae	Aquilegia	vulgaris	Chito 02
1377	2	Ranunculaceae	Aquilegia	vulgaris	MeJa 03
1379	3	Lamiaceae	Leonurus	sibiricus	control 01
1381	3	Lamiaceae	Leonurus	sibiricus	Acetate 03

1383	2	Lamiaceae	Leonurus	sibiricus	AgN03 02
1385	0	Lamiaceae	Leonurus	sibiricus	Chito 02
1387	1	Lamiaceae	Leonurus	sibiricus	MeJa 03
1395	1	Ephedraceae	Ephedra	nevadensis	Acetate 03
1397	3	Convolvula-ceae	Ipomoea	purpurea	control 01
1399	3	Convolvula-ceae	Ipomoea	purpurea	Acetate 03
1401	2	Convolvula-ceae	Ipomoea	purpurea	AgN03 02
1403	2	Convolvula-ceae	Ipomoea	purpurea	Chito 02
1405	3	Convolvula-ceae	Ipomoea	purpurea	MeJa 03
1407	3	Arnarantha-ceae	Cyathula	officinalis	Acetate 03
1409	3	Asteraceae	Xanthium	sibiricum	Acetate 03
1413	3	Fabaceae	Tephrosia	grandiflora	Acetate 03

Table V: Sniffing test on cuticular washings

sample #	rating	family	genus	species
wx 201	1	Rutaceae	Murrays	exotica
wx 202	2	Araliaceae	Trevesia	sungaica
wx 203	1	Verbenaceae	Clerodendrum	speciosissimum
wx 204	0	Euphorbiaceae	Acalypha	hispida
wx 205	2	Gnetaceae	Gnetum	gnemon
wx 206	1	Rubiaceae	Psychotria	nigropunctata

wx 207	1	Rubiaceae	Psychotria	metbacterio-domasica
wx 208	2	Euphorbiaceae	Codiaeum	variegatum
wx 209	2	Euphorbiaceae	Phyllanthus	grandifolius
wx 210	3	Sterculiaceae	Pterigota	alata
wx 211	1	Bombacaceae	Pachira	affinis
wx 212	1	Sterculiaceae	Sterculia	elata
wx 213	0	Araceae	Philodendron	speciosum
wx 214	2	Fabaceae	Pithecellobium	unguiscati
wx 215	1	Acanthaceae	Sanchezia	nobilis
wx 216	1	Araliaceae	Oreopanax	capitata
wx 217	0	Moraceae	Ficus	triangularis
wx 218	2	Bignoniaceae	Kigelia	pinnata
wx 219	1	Piperaceae	Piper	cubeba
wx 220	3	Lauraceae	Laurus	nobilis
wx 221	2	Fabaceae	Erythrinia	cristagalli
wx 222	1	Myrataceae	Metrosideros	excelsa
wx 223	3	Oleaceae	Osmanthus	fragrans
wx 224	1	Cupressaceae	Tetraclinis	articulata
wx 225	0	Cupressaceae	Cupressus	sempervirens
wx 226	0	Acanthaceae	Jacobinia	sp.
wx 227	0	Asteraceae	Senecio	platifilla
wx 228	1	Arecaceae	Livistona	chinensis
wx 229	3	Myrataceae	Eucalyptus	rudis
wx 230	1	Podocarpaceae	Podocarpus	spinulosus
wx 231	2	Rosaceae	Eriobotrya	japonica
wx 232	2	Ginkgoaceae	Gingko	biloba
wx 233	2	Ericeae	Rhododendron	sp.
wx 234	2	Cupressaceae	Thuja	occidentalis
wx 235	1	Polygonaceae	Fagopyrum	suffruticosum

wx 236	1	Rosaceae	Geum	macrophyllum
wx 237	2	Magnoliaceae	Magnolia	cobus
wx 238	3	Apocynaceae	Vinca	minor
wx 239	0	Liliaceae	Convallaria	majalis
wx 240	2	Betulaceae	Corvlus	avellana
wx 241	2	Berberidaceae	Berberis	sp.
wx 242	2	Rosaceae	Rosa	multiflora
wx 243	1	Betulaceae	Ostrya	carpinifolia
wx 244	1	Betulaceae	Ostrya	connogea
wx 245	1	Fagaceae	Quercus	rubra
wx 246	2	Magnoliaceae	Liriodendron	tulipifera
wx 247	1	Rosaceae	Sorbus	aucuparia
wx 248	3	Betulaceae	Betula	nigra
wx 249	3	Betulaceae	Betula	nigra
wx 250	2	Fagaceae	Castanea	sativa
wx 251	0	Saxofragaceae	Bergenia	crussifolia
wx 252	3	Asteraceae	Artemisia	dracunculus
wx 253	3	Rutaceae	Ruta	graveolens
wx 254	3	Fragaceae	Quercus	nigra
wx 255	2	Schisandraceae	Schisandra	chinensis
wx 256	3	Betulaceae	Betula	alba
wx 257	2	Caprifoliaceae	Sambucus	nigra
wx 258	3	Actinidiaceae	Actinidia	colonicta
wx 259	2	Paeoniaceae	Paeonia	lactiflora
wx 260	1	Paeoniaceae	Paeonia	suffructicisa
wx 261	0	Fragaceae	Quercus	imbricaria
wx 262	1	Iridaceae	Iris	pallida
wx 263	2	Portulacaeae	Portulaca	oleracea
wx 264	2	Polygonaceae	Polygonum	aviculare

wx 265	0	Iridaceae	Iris	pseudacorus
wx 266	1	Liliaceae	Allium	nutans
wx 267	3	Liliaceae	Allium	fistulosum
wx 268	1	Liliaceae	Anthericum	ramosum
wx 269	1	Liliaceae	Veratrum	nigrum
wx 270	1	Polygonaceae	Polygonum	latifolia
wx 271	0	Liliaceae	Hosta	lancifolia
wx 272	1	Liliaceae	Hosta	zibalda
wx 273	2	Asteraceae	Echinops	sphaerocephalus
wx 274	3	Paeoniaceae	Paeonia	daurica
wx 275	1	Asteraceae	Inula	helenium
wx 276	2	Fabaceae	Crambe	pontica
wx 277	0	Scrophuylar-iaceae	Digitalis	lutea
wx 278	3	Fabraceae	Baptisia	australis
wx 279	2	Aristolochia-ceae	Aristolochia	clematitis
wx 280	3	Lamiaceae	Hyssopus	zeraucharicus
wx 281	2	Lamiaceae	Teucrium	hamedris
wx 282	2	Crassulaceae	Sedum	album
wx 283	3	Apiaceae	Heracleum	pubescens
wx 284	3	Lamiaceae	Origanum	vulgare
wx 285	3	Apiaceae	Cachrys	alpina
wx 286	1	Apiaceae	Laser	trilobum
wx 287	2	Dryopteridaceae	Matteucia	struthiopteris
wx 288		Crassulaceae	Sedum	telephium
wx 289	2	Papaveraceae	Bocconia	cordata
wx 290	1	Lamiaceae	Ajuga	reptans
wx 291	1	Ranunculaceae	Thalictrum	minus
wx 292	2	Ranunculaceae	Anemone	japonica

wx 293	1	Ranunculaceae	Clematis	recta
wx 294	2	Ranunculaceae	Thalictrum	sp.
wx 295	1	Rosaceae	Alchemilla	sp.
wx 296	1	Rosaceae	Potentilla	alba
wx 297	1	Rosaeae	Poterium	sangiusorba
wx 298	3	Menispermaceae	Menispermum	dauricum
wx 299	3	Nyctaginaceae	Oxybaphus	nyctagineus
wx 300	2	Brassicaceae	Armoracia	rusticana

The results presented hereinabove, demonstrate that plant exudates and components of cuticular coatings constitute a novel and important source of new biologically active compounds having antimicrobial, antifungal, insecticidal, sporidicidal, cytotoxic activities and herbicidal properties that could be used for treatments of various diseases or conditions. In addition, compounds present in cuticular washings and root exudates can be used as fragrances, flavors, and flavor enhancers.

EXAMPLE 3

Phytosecretion of Genistein and Daidzein by Soybean Roots

Soybean *(Glycine max)* seeds were germinated in a greenhouse equipped with supplementary lighting (16-h photoperiod 24-28°C). Seeds were placed inside 0.9 cm diameter, 0.9 cm deep well drilled in Grodan rockwool cubes *(3.4* cm width x *3.4* cm depth x 3.7 cm height) purchased from Grodania A/S, Hedehousene, Denmark.

Depending on the speed of germination, the seeds were either placed directly into the rockwool cubes or sterilized to prevent rotting during the germination process. For sterilization, seeds were immersed first in 70% ethyl-Alcohol for 10-15 seconds, then in 2.5% Sodium

Hypochlorite for 10-15 min., and finally rinsed thoroughly with distilled water. The sterilized seeds were placed in a Petri dish lined with no. 1 Wattman paper (Wattman International Ltd., Maidstone, England), soaked in either a sterile water for seeds larger than 1 mm in diameter, or for smaller seeds with mineral salts nutrient solution. The Petri-dishes were sealed with parafilm before being placed in a growth chamber (12-h photoperiod 22-24°C) until the seeds germinated.

Rockwool cubes were placed inside standard greenhouse plastic trays (dimensions 52 cm width x 25 cm depth x 7 cm height) and watered with an intermittently operating overhead misting system triggered by a moisture sensor (Mist-A-Matic, E.C. Geiger Inc., Larleysville, PA). Seeds were allowed to germinate for 3-6 days till the roots started to emerge from the bottom of-the rockwool cube.

After germination, the cubes with the seedlings were inserted into a 3.2 cm diameter round opening cut in the center of Styrofoam ring (8.2 cm diameter, 2.5 cm thickness). The ring was floated on the surface of 400-800 mL of hydroponic nutrient solution (2 g/L Hydro-Sol (Scotts-Sierra Horticultural Products Comp., Marysville, OH] supplemented with 1.2 g/L $Ca[NO_3]_2$) containing inside light impermeable, high-density polyethylene cylinder (9.0 cm in diameter, 16 cm in height).

Aeration was provided either by shaking the cylinders at 50 rpm on the platform shaker (Model Orbit, Lab-Line Instruments, Inc., Melrose Park, IL) or by bubbling compressed air through the solution. Seedlings were cultivated hydroponically in this system for 3 to 5 weeks with roots growing in a nutrient solution. Thereafter, the root system (average root dry weight

0.1±0.05g) was removed from the nutrient solution and placed inside a 30 mL glass beaker, containing 10-20 mL of distilled water or distilled water supplemented with the elicitor. To prevent the water loss from the plant canopy and the drying of the collecting solution, shoots of the plants were covered with a plastic bag. After 24 h, unless noted otherwise, a small sample from the root solution was removed and analyzed for the phytosecreted products.

EXAMPLE 4
Flow-Through Phytosecretion System

The flow-through phytosecretion system consisted of a stainless steel container (53 cm width x 34 cm depth x 20 cm height) with 15-24 soybean plants supported by the rockwool cubes inserted in the openings in the Styrofoam raft (5.0 cm thickness) which had dimensions slightly smaller than the internal dimensions of the container. This Styrofoam raft was floating on top of approximately 10 L of nutrient solution (2g/L Hydro-Sol supplemented with 1.2 g/L Ca $(NO_3)_2$), aerated with compressed air supplied through an air hose placed on the bottom of the container. After 4-5 weeks, or when the roots reached the appropriate size, the volume of nutrient solution was reduced to 2L. The flow of the nutrient solution, with or without an elicitor, through the flow through system was maintained with a peristaltic pump (Variable Flow Mini-Pump, Fisher Scientific, Pittsburgh, PA), which allowed easy adjustments in the volume of the solution entering the system. Typically, flow rates used in the experiments ranged from 1.5 to 4.5 L/day. The intake tube of the peristaltic pump was immersed in a 60 L plastic storage container containing

nutrient solution. Solution from the storage container dripped into the phytosecretion system through the tube attached to its wall. When necessary, elicitors were added to the storage container at the desired concentration. The solution was discharged from the phytosecretion container in the side opposite to the inlet through the opening cut in the bottom of the container. Solution level in the phytosecretion container was adjusted by changing the height of the opening of the outlet tube. Solution samples were taken from the end of the outlet tube at the specified intervals and analyzed for the presence of the phytosecreted compounds.

EXAMPLE 5

High-Pressure Liquid Chromatography (HPLC) Analysis of Phytosecreted Natural Products (Isoflavonoids)

An HPLC method for separation and identification of phytosecreted compounds, using isoflavonoids daidzein and genistein is used as an example. The chromatography separation was performed with an HPLC-system consisting of Waters 996 Photodiode Array Detector (PDA) with usable UV range from 190 to 800 nm; a Waters (xxx) 717 plus autosampler; two Beckman (xxx) 110B solvent Delivery Modules, connected with a Beckman System Organizer (mixer) and a Beckman System Gold Analog Interface Module 406. The Beckman solvent delivery system was controlled by a NEC PC-8300 computer. Chromatography and spectral data was managed by Waters Millennium chromatography software, version 2.10, using a NEC Image 466es computer. All hardware components, except the solvent delivery system, were connected through a standard IEEE communication system.

Isoflavonoid compounds were separated on a Waters Nova Pak® C-18 reverse phase column, 3.9 x 150 mm, 60Å pore size, and 4 μm particle size.

The mobile phase consisted of two components: Solvent A - 0.5% ACS grade acetic acid in double distilled water, pH 3-3.5; and Solvent B - acetonitrile. Prior to use, each batch of solvent A was degassed under vacuum and ultrasonication for 5 minutes.

The mobile phase flow was adjusted to 1 ml/min, and a gradient mode of separation was used for all separations. The gradient profile was as follows:

0 - 20 min 0% B - 100% B;

20 - 22 min 100% B;

22 - 25 min 100% B - 0% B;

25 - 33 min 0% B (column equilibration for next injection).

Compounds were detected with PDA detector within the wavelength range of 200 to 400 nm. The column temperature was ambient.

Under the above conditions, daidzein had RT 11.725 and UV maxima at 250.9 nm and 302.9 nm and genistein had a RT of 12.94 min and UV maximum at 260.3 nm. Depending on the resolution setting of the PDA detector, a negligible shift of $\pm$3 nm in the absorbance maxima was observed. A$\pm$0.5 min tolerance in the retention times with the different batches of solvents was detected.

All plants were grown hydroponically, as previously described, and phytosecreted compounds collected for 24 hours in distilled water containing an elicitor or mixtures of different elicitors, except for treatment 47, where no elicitors were present in the collecting water. Daidzein and genistein content in root

exudates from unelicited plants grown under the same conditions was below the detection limits - 400 pg, or 4 μg/L for daidzein, and 25 pg, or 250 ng/L for genistein.

Elicitor treatments:

3 - Salicylic acid (5 mM) , Tetcyclases (0.2 mM) and 7.5% EtOH

5 - Salicylic acid (5 mM) and 5% EtOH

6 - Salicylic acid (2.5 mM) and 2.5% EtOH

7 - Salicylic acid (5 mM) and 0.5 g/L SDS

10 - Salicylic acid (1 mM) and Pentafluorobenzoic acid(2 mM)

21 - Silver nitrate (1 mM) in acidic pH (citric acid, pH 2.7)

24 - Silver nitrate (2 mM) in acidic pH (acetic acid, pH 2.7)

25 - Acetic acid (pH 2.7)

37 - Pentafluorobenzoic acid (5 mM)

38 - 2.6 - Dihydroxybenzoic acid (10 mM)

40 - Cinnamic acid (16.5 mM) and 35.5% EtOH

42 - Cinnamic acid (3.3 mM) and 7.1% EtOH

45 - 2-Fluorobenzoic acid (10 mm) and 2% EtOH

47 - UV-light irradiation of the whole plant for 3 hours

55 - Sodium,-fluoride (250 mM) and 10% EtOH

Fifteen treatments which elicited some of the highest levels of daidzein and genistein are shown in Figures 16 and 17 for simplicity. Some of the above elicitors induced mild to moderate phytotoxicity in the treated plants. All compounds used in the mixtures produced significant levels of daidzein and genistein, when applied alone. However, combinations of various elicitors shown in Figures 16 and 17 usually produced

higher levels of target compounds. Other compounds used as elicitors, such as yeast extract, laminarin, SDS, jasmonic acid, methyl jasmonate, okadaic acid, polygalacturonic acid, 1-phosphatidic acid, polyethylene glycol, hydrogen peroxide, paraquate, calyculin A, 1-amino butyrate, eicosapentaenoic acid, arachidonic acid, glutathione, ascorbic acid, and some heavy metals (Nickel, Copper, Lead) showed lower degree of elicitation of the target compounds.

Various plants were grown hydroponically as previous described and secreted compounds (root exudates) were collected in distilled water with and without an elicitor(s).

Figure 18 is an HPLC profile of compounds recovered from the root exudates with UV detection at 251.8 nm. Most compounds were not identified, however, as shown in Figure 18 the following designations in Figure 18 were positively or putatively identified.

1. Positively identified Daidzein
2. Positively identified Genistein

A. Putatively identified as nicotine

B. Putatively identified as (5-0-methyl-genistein)

Lupinus luterus roots were not elicited; *Lupinus polyphyllus* roots were elicited with 2 mM Salicylic acid in 2% Ethanol; all other species were elicited with 0.3 M Acetic acid, pH 2.7.

Figure 19 demonstrates the diversity of compounds excreted from the roots of one plant species (*Lupinus luterus*) treated-with different elicitors (note the large differences in the HPLC profiles of root exudates produced by different elicitors), UV detection at 251.8 nm.

Elicitor treatments:

control - No treatment

I - Treatment with 2 mM Salicylic acid in 2% Ethanol

II - Treatment with 0.3 M Acetic acid, pH - 2.7

III - Treatment with 2 mM AgN03

IV - Treatment with 7.5 mM Arachidonic acid

V. - Treatment with 5 mM Jasmonic acid

Most compounds were not identified. Those positively or putatively-identified are:

1 - Putatively identified as 5-0-Methyl-genistein

G - Positively identified as Genistein

SA - Salicylic acid

Figures 20, 21, 22 and 23 are HPLC profiles of the diversity of compounds exuded or leached from the roots of *Brassica juncea, Datura metel, Lupinus polyphyllus* and *Melilotus medicaginoides*, respectively, treated with different elicitors. UV detection was at 254 nm. No compound was identified.

The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification within the scope of the appended claims.

What is Claimed:

1. A method of identifying an agent exuded onto the surface of a leaf of a plant having biological activity, the method comprising:

(a) removing cuticular material located on the surface of a leaf of the plant, comprising contacting the leaf surface with a solvent, thereby resulting in a solvent solution;

(b) assaying the solvent solution for biological activity; and

(c) analyzing the solvent solution so as to identify the agent which has the biological activity.

2. The method of claim 1, wherein the biological activity is antimicrobial, insecticidal, or herbicidal, fragrances, scent, flavors, and flavor enhancers.

3. The method of claim 2, wherein the antimicrobial activity is antibacterial or antifungal activity.

4. The method of claim 1, wherein the step of assaying the solvent solution comprises contacting the solution its components with a media containing a suspension of a microorganism, wherein the inhibition of the growth of the suspension of the microorganism is indicative of an agent in the solvent solution having biological activity.

5. The method of claim 4, wherein the media is

a liquid medium or an agar media.

6. The method of claim 1, wherein the cuticular material is a lipid, wax, cutin, protein, primary or secondary metabolite.

7. The method of claim 6, wherein the cuticular material is a wax.

8. The method of claim 1, wherein the solvent is an organic solvent.

9. The method of claim 8, wherein the organic solvent is methylene chloride or chloroform.

10. The method of claim 1, wherein the suspension of microorganism is a bacteria, fungus, or virus.

11. The method of claim 10, wherein the suspension of microorganism is selected from the group consisting of *Escherichia coli* K- 12. F, prototropic Str., *Staphylococcus aureus subsp. Aureus, Pseudomonas aeruginosa, Saccharomyces cerevisiae, Aspergillus flavus* and *Penicillium nigra.*

12. The method of claim 1, wherein the plant is a higher plant.

13. The method of claim 12, wherein the plant is selected from a group consisting of *Atropa belladonna, Erythrinia glabeliferus, Ipomea tricolor, Erythrinia crista, Celosia cristata, Gallium sporium, Laurus*

nobilis, Vitis labrissa, Gratiola officinalis, Symphitum officinalis, Hosta fortuna, Casia hebecarpa, Thalictum flavum, Scutellarian altissima, Portulaca oleacea, Scutellaria certicola, Physalis creticola, Geum fanieri, Gentiana tibetica, Linum hirsutum, Aconitum napellus, Podophyllum amodii, Thymus cretaceus, Carlina acaulis, Chamaechrista fasciculata, Pinus pinea, Pegamun hamalis, Tamarindus india, Carica papaya, Cistus incanus, Capparis spinosa inemis, Cupress lusitanica, Diopiros kaka, Erungiurn campestre, Aesculus woerlitzenis, Aesculus hippocastanum, Cupressus sempervirens, and *Celtis occidentalis.*

14. The method of claim 1, wherein step of I (c) furthers comprises fractioning the solvent solution.

15. The method of claim 14, furthers comprising identifying the agents.

16. A method of identifying an agent exuded onto roots of a plant having biological activity, the method comprising:

(a) obtaining a sample of a aqueous media after it contacted plant roots;

(b) assaying the sample for biological activity; and

(c) analyzing the sample so as to identify the agent which has the biological activity.

17. The method of claim 16, wherein the biological activity is antimicrobial or herbicidal, fragrances, scent, flavors, and flavor enhancers.

18. The method of claim 17, wherein the antimicrobial activity is antibacterial or antifungal activity.

19. The method of claim 16, wherein the step of assaying the sample comprises contacting the solution onto a medium having thereon a suspension of a microorganism, wherein the inhibition of the growth of the suspension of the microorganism is indicative of an agent in the solvent solution having biological activity.

20. The method of claim 16, wherein the suspension of microorganism is a bacteria, fungus, or virus.

21. The method of claim 20, wherein the suspension of microorganism is selected from the group consisting of *Escherichia coli* K-12. F, prototropic Str., *Staphylococcus aureus subsp. Aureus, Pseudomonas aeruginosa, Saccharomyces cerevisiae, Aspergillus flavus* and *Penicillium nigra*.

22. The method of claim 16, wherein the plant is a higher plant.

23. The method of claim 22, wherein the plant is selected from a group consisting of *Atropa belladonna, Erythrinia glabeliferus, Ipomea tricolor, Erythrinia crista, Celosia cristata, Gallium sporium, Laurus nobilis, Vitis labrissa, Gratiola officinalis, Symphitum officinalis, Hosta fortuna, Casia hebecarpa, Thalictum flavum, Scutellarian altissima, Portulaca oleacea, Scutellaria certicola, Physalis creticola, Geum fanieri,*

Gentiana tibetica, Linum hirsutum, Aconitum napellus, Podophyllum amodii, Thymus cretaceus, Carlina acaulis, Chamaechrista fasciculata, Pinus pinea, Pegamun hamalis, Tamarindus india, Carica papaya, Cistus incanus, Capparis spinosa inemis, Cupress lusitanica, Diopiros kaka, Erungiurn campestre, Aesculus woerlitzenis, Aesculus hippocastanum, Cupressus sempervirens, and *Celtis occidentalis.*

24. The method of claim 16, wherein the method further comprises adding an elicitor to the aqueous media contacting the roots prior to the removal of the roots from the media.

25. The method of claim 24, wherein the method further comprises adding an elicitor to the aqueous media which contact the roots during the growth of the roots.

26. The method of claim 24, wherein the elicitor is an abiotic or biotic elicitor.

27. The method of claim 26, wherein the biotic elicitor is chitosan, fungal, bacterial cell wall, methyl salicylate, or methyl jasmonate.

28. The method of claim 26, wherein the abiotic elicitor is silver nitrate or acetic acid.

29. A method for recovering substances exuded or secreted from a plant or plant part, which comprises contacting a living plant or plant part with an aqueous medium to extract into the medium the substances exuded by the plant or plant part, and thereafter recovering the

exuded substances from the aqueous medium.

30. The method of claim 29, wherein the plant is subjected to a physical or chemical treatment to induce production of novel substances or to increase production of a pre-determined substance, prior to or during the contacting with the aqueous medium.

31. The method of claim 29, wherein the aqueous medium is continuously or semi-continuously brought into contact with the plant or plant part.

32. A method for generating a library of plant-exuded substances for subsequent screening, which comprises contacting a living plant or plant part with an aqueous medium to extract into the medium the substances exuded by the plant or plant part, recovering the exuded substances from the aqueous medium and forming a library of the substances recovered from the aqueous medium.

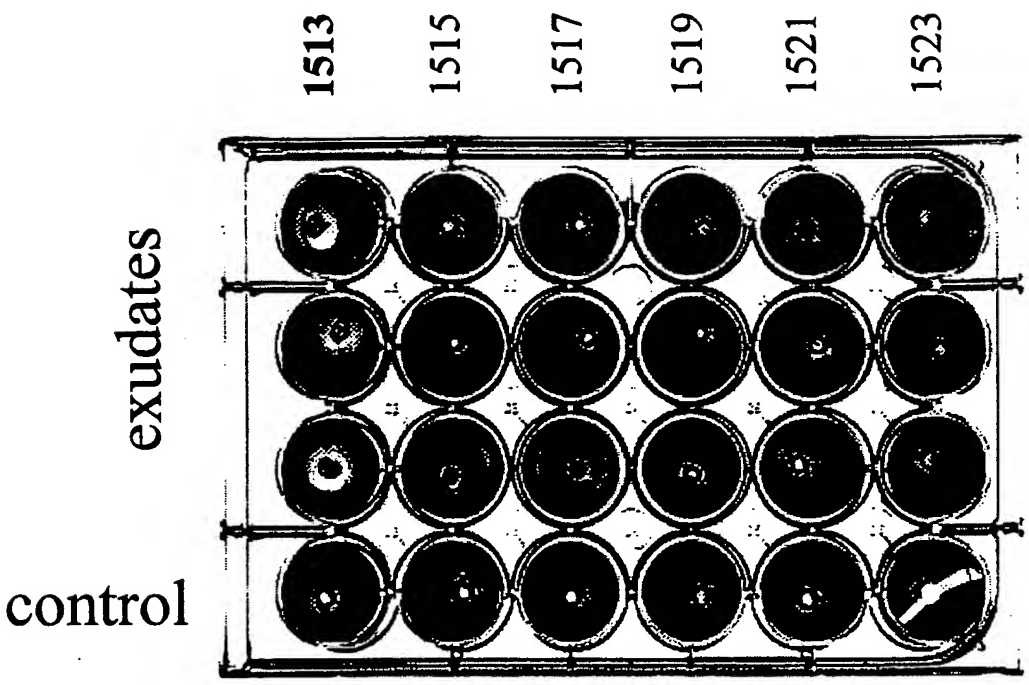


Figure 1

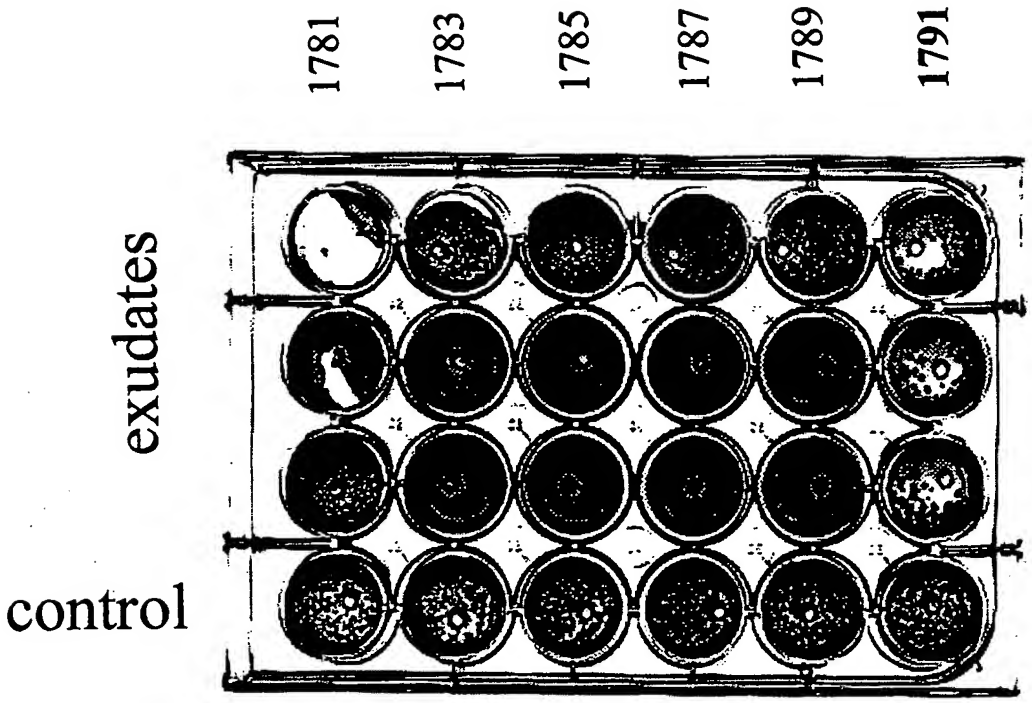


Figure 2

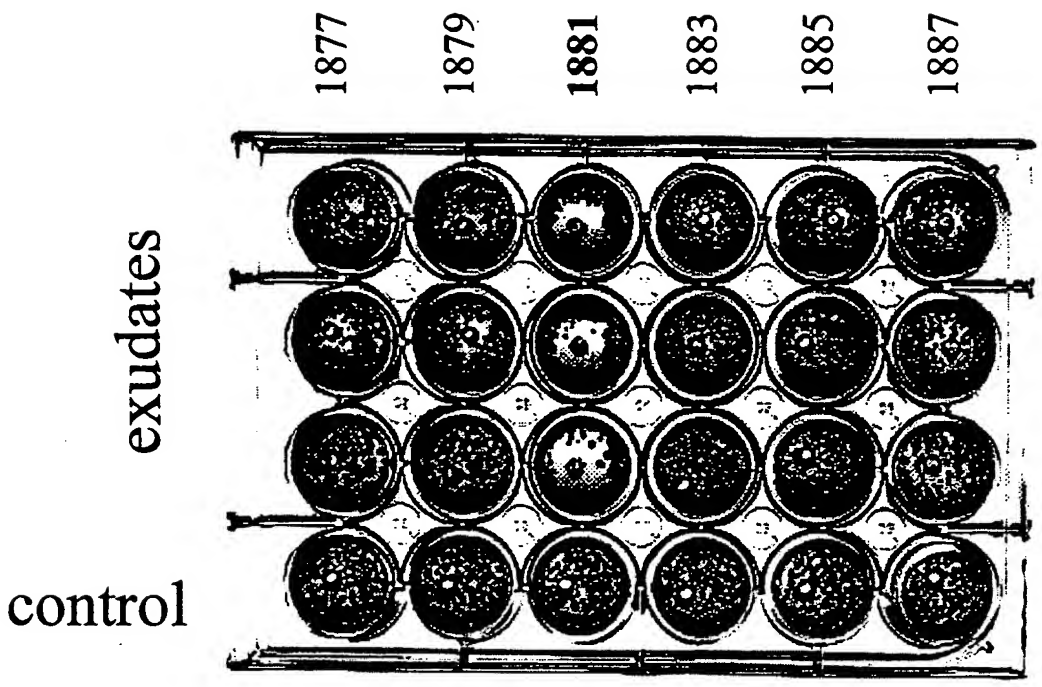


Figure 3

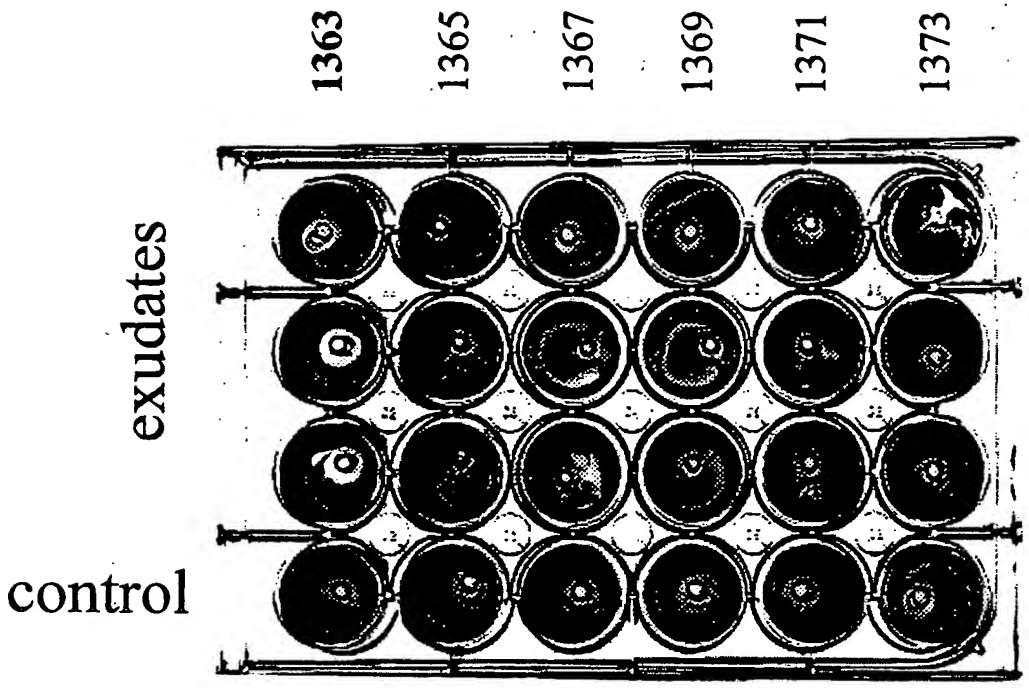


Figure 4

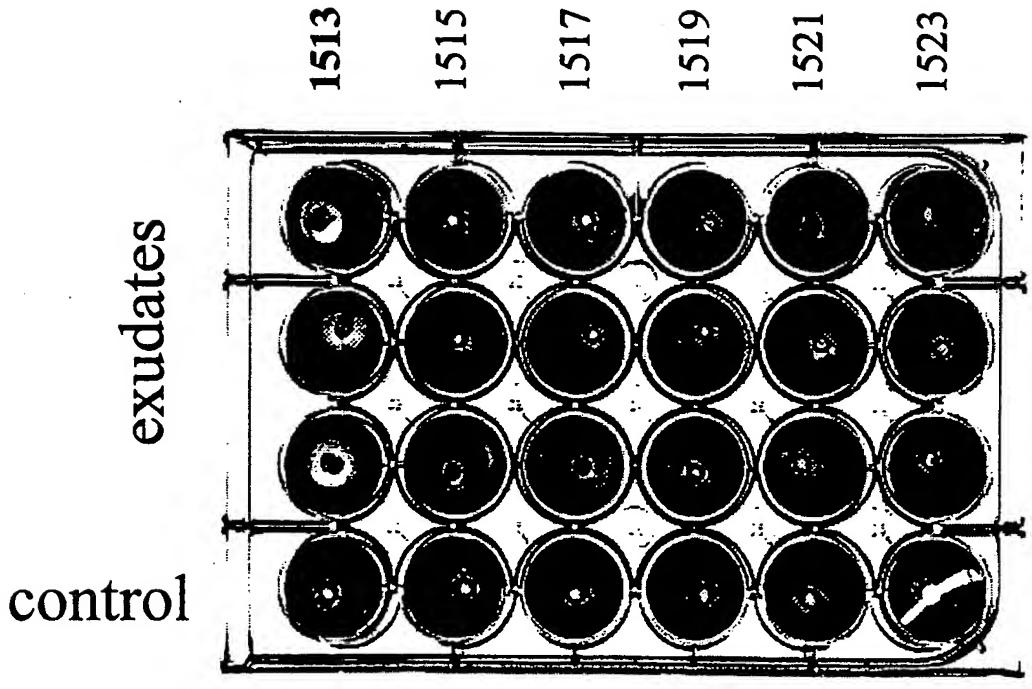


Figure 5

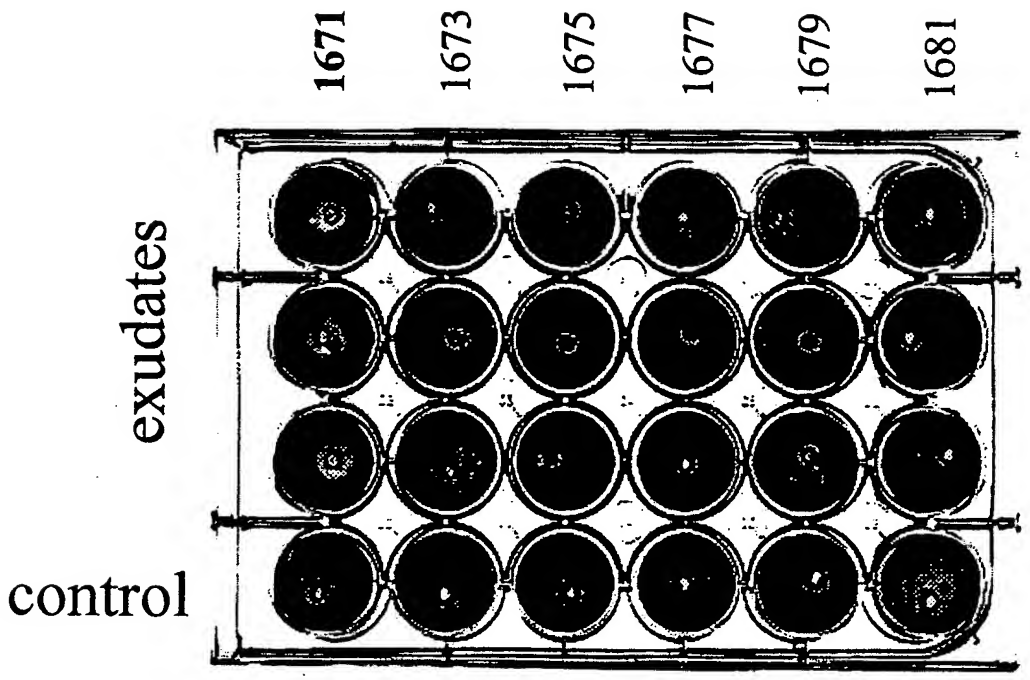


Figure 6

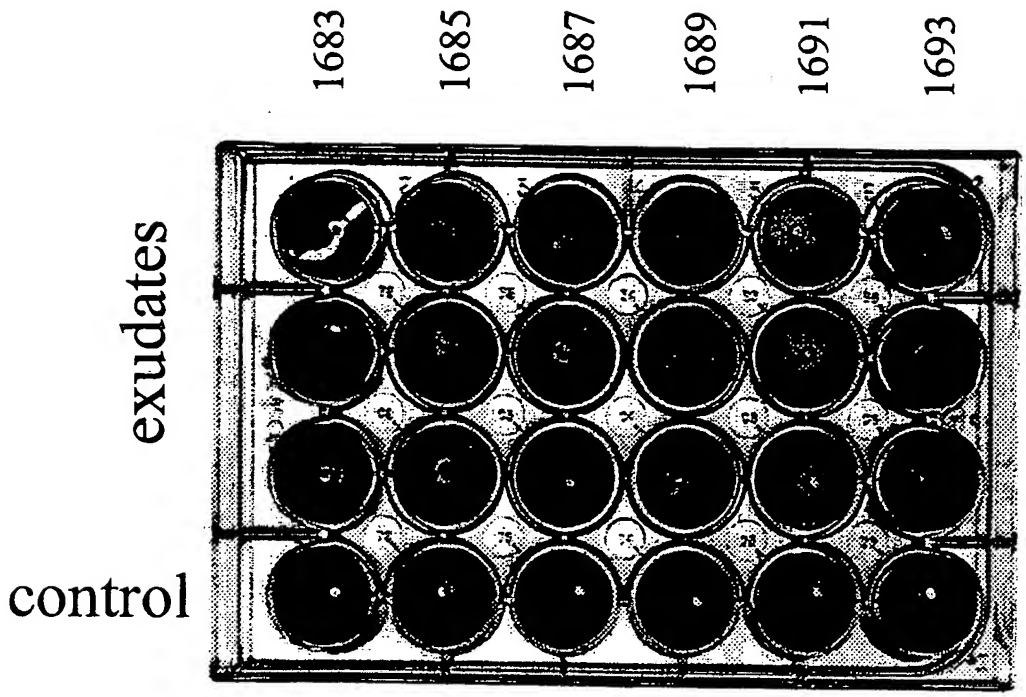


Figure 7

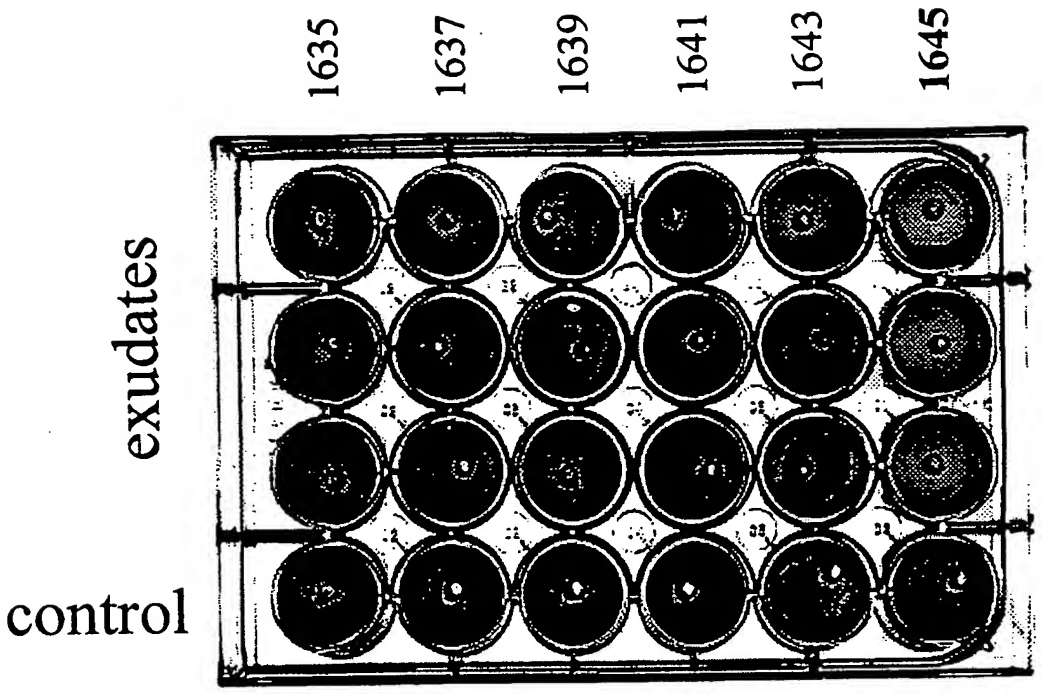


Figure 8

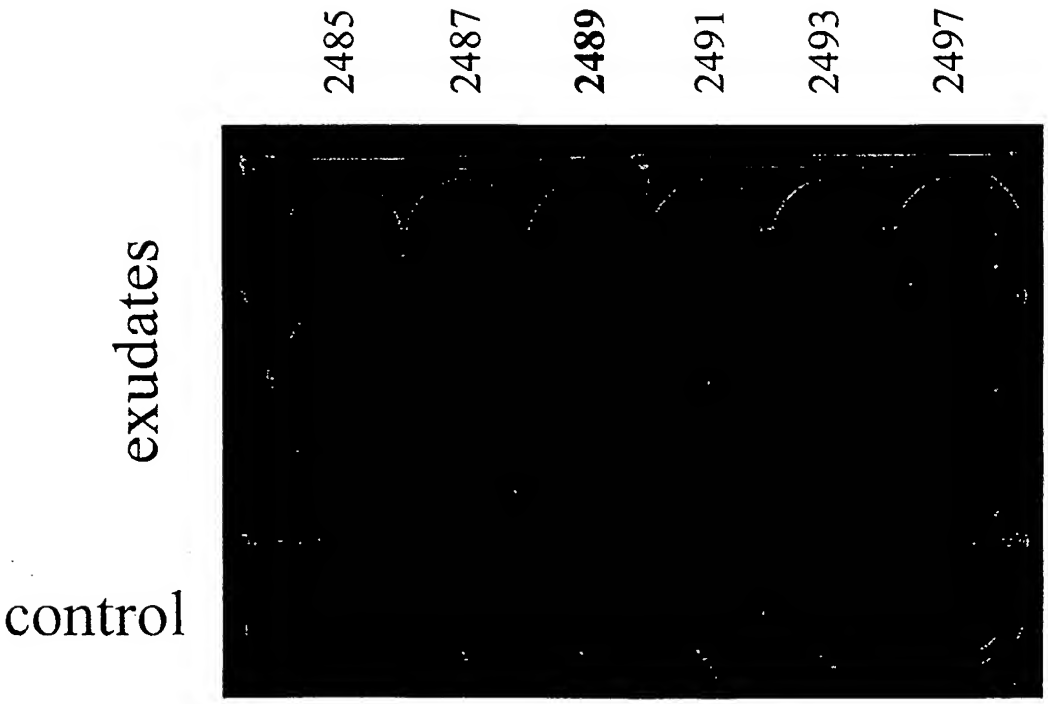


Figure 9

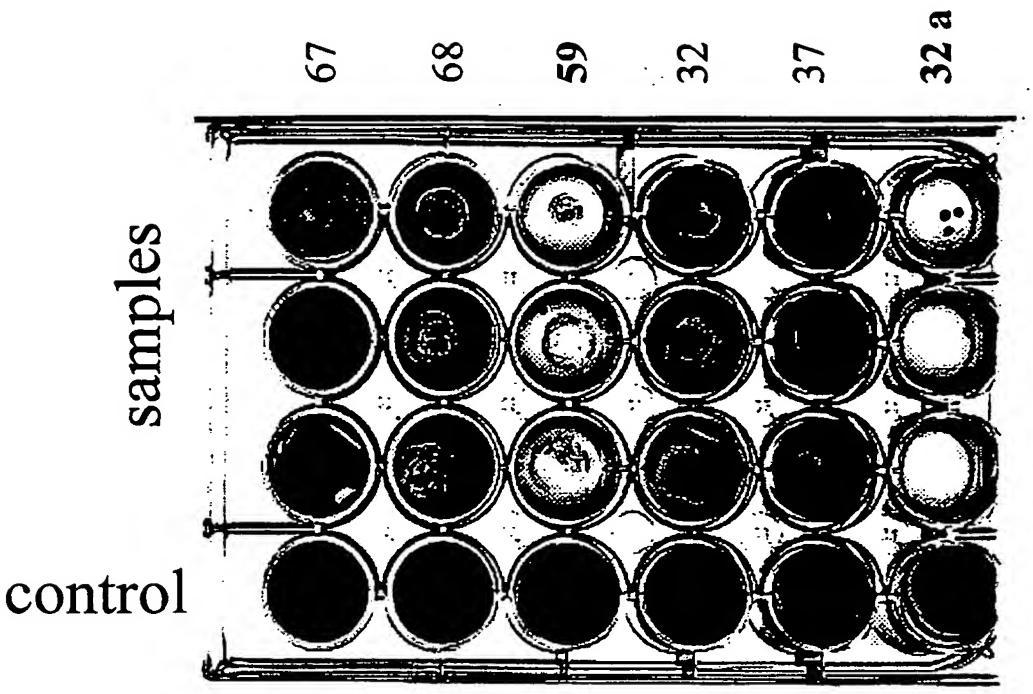


Figure 10

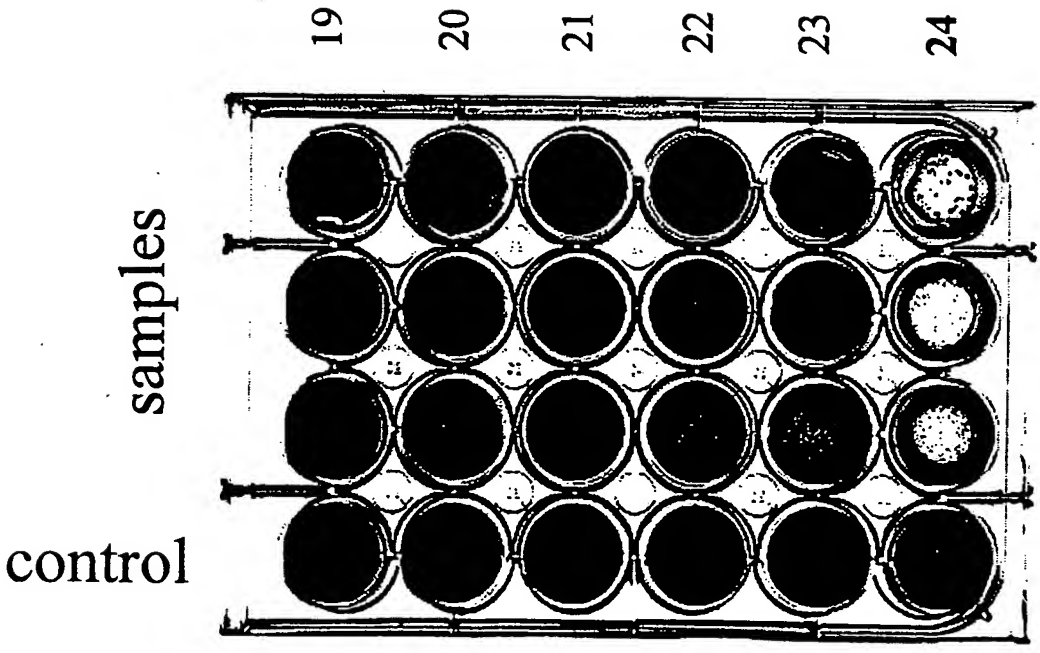


Figure 11

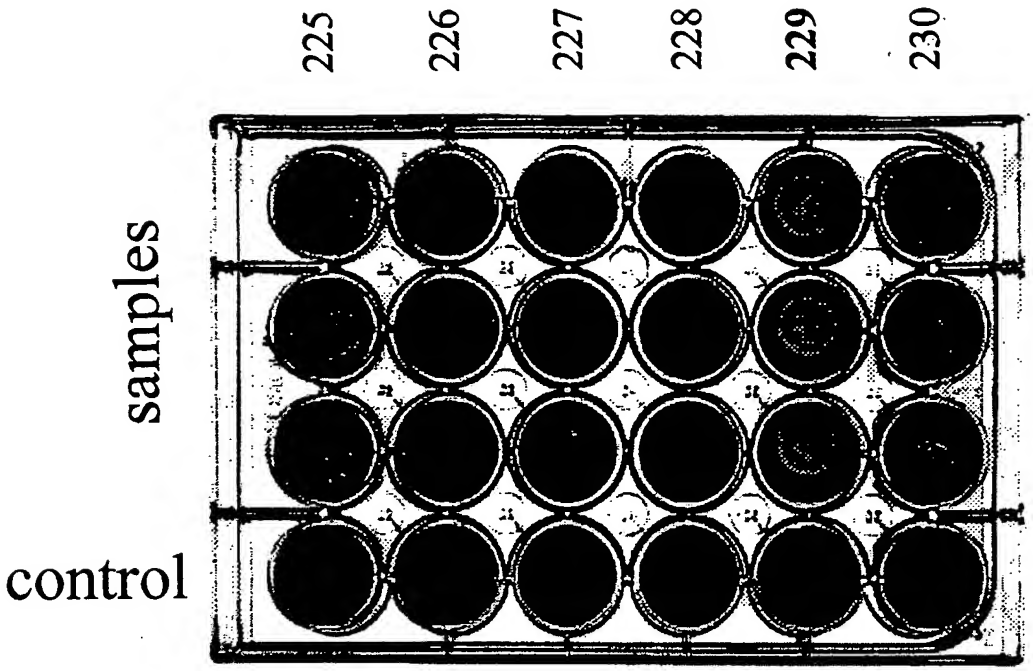


Figure 12

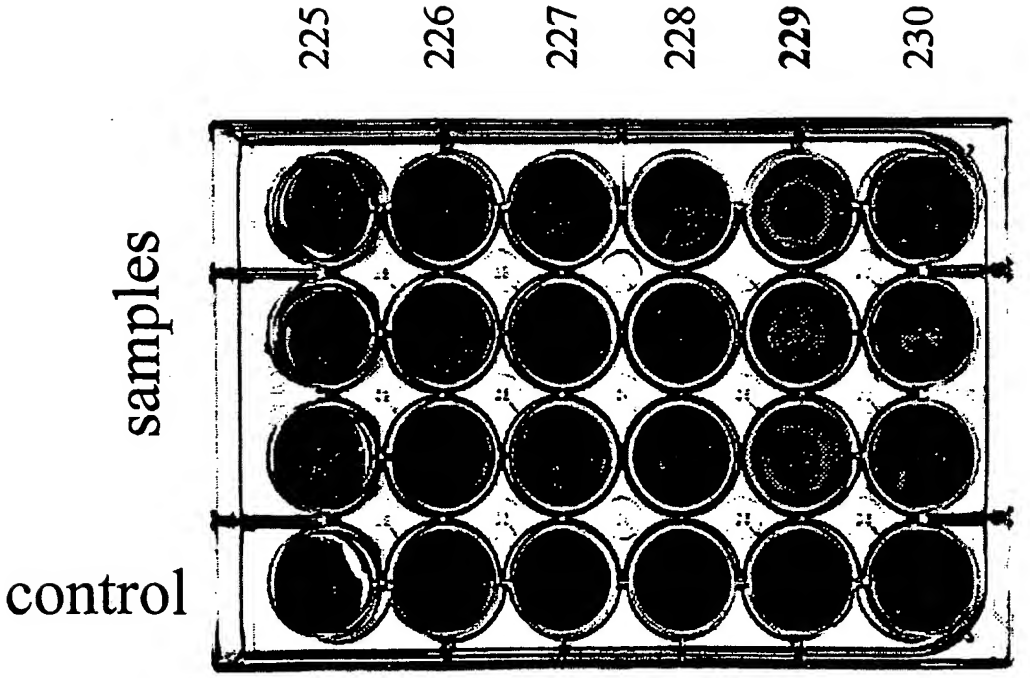


Figure 13

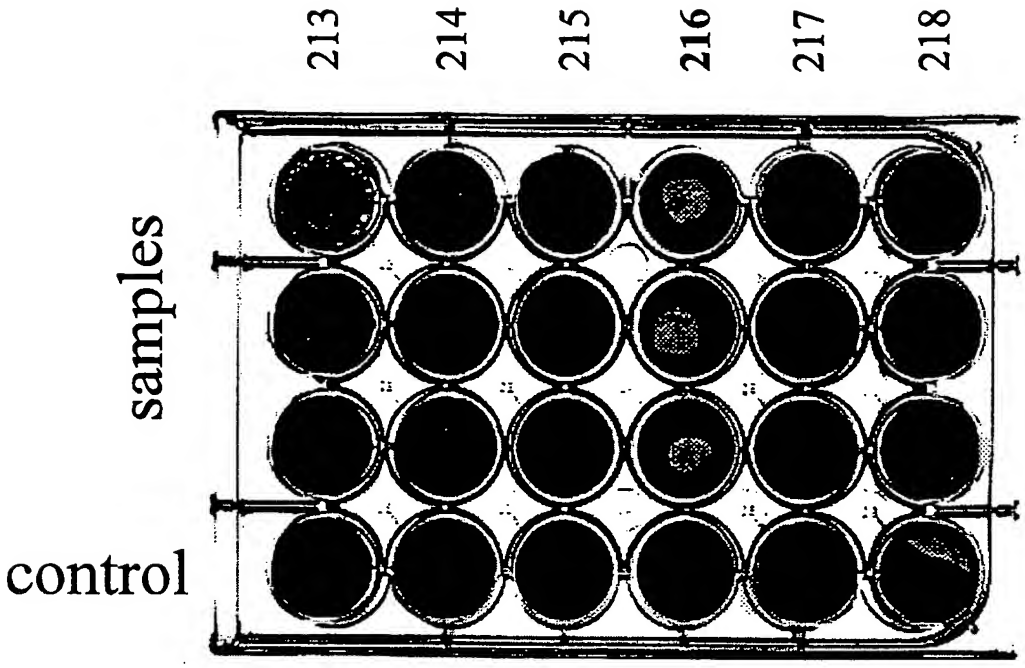


Figure 14

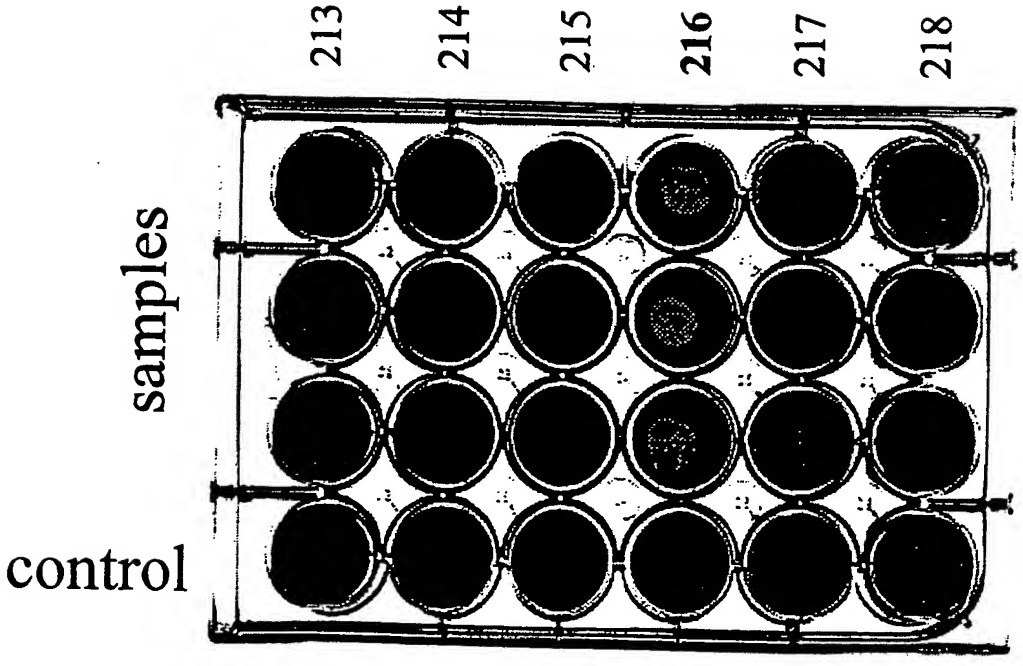


Figure 15

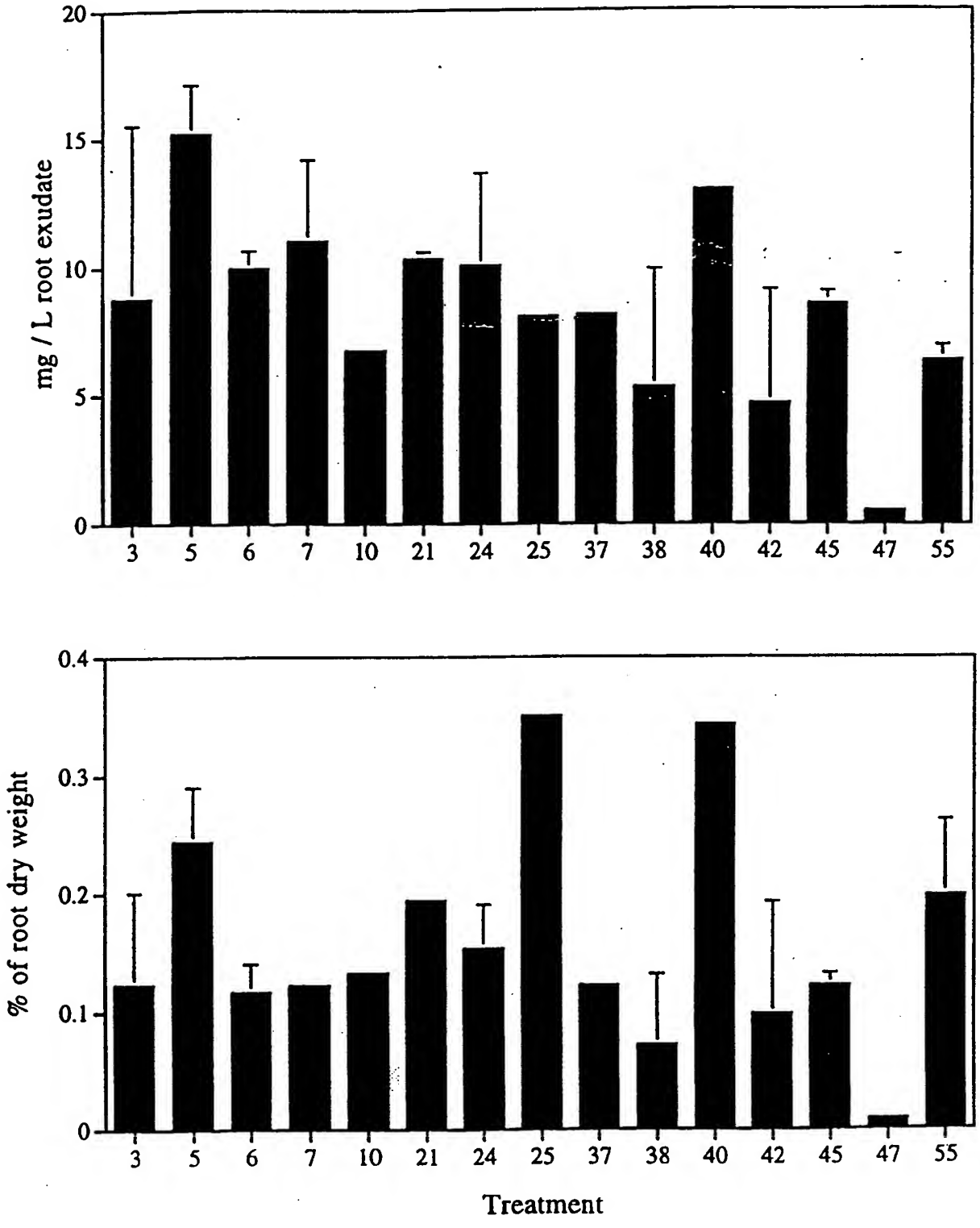

mg / L root exudate
20
15
10
5
0
3 5 6 7 10 21 24 25 37 38 40 42 45 47 55
% of root dry weight
0.4
0.3
0.2
0.1
0
3 5 6 7 10 21 24 25 37 38 40 42 45 47 55
Treatment

Figure 16

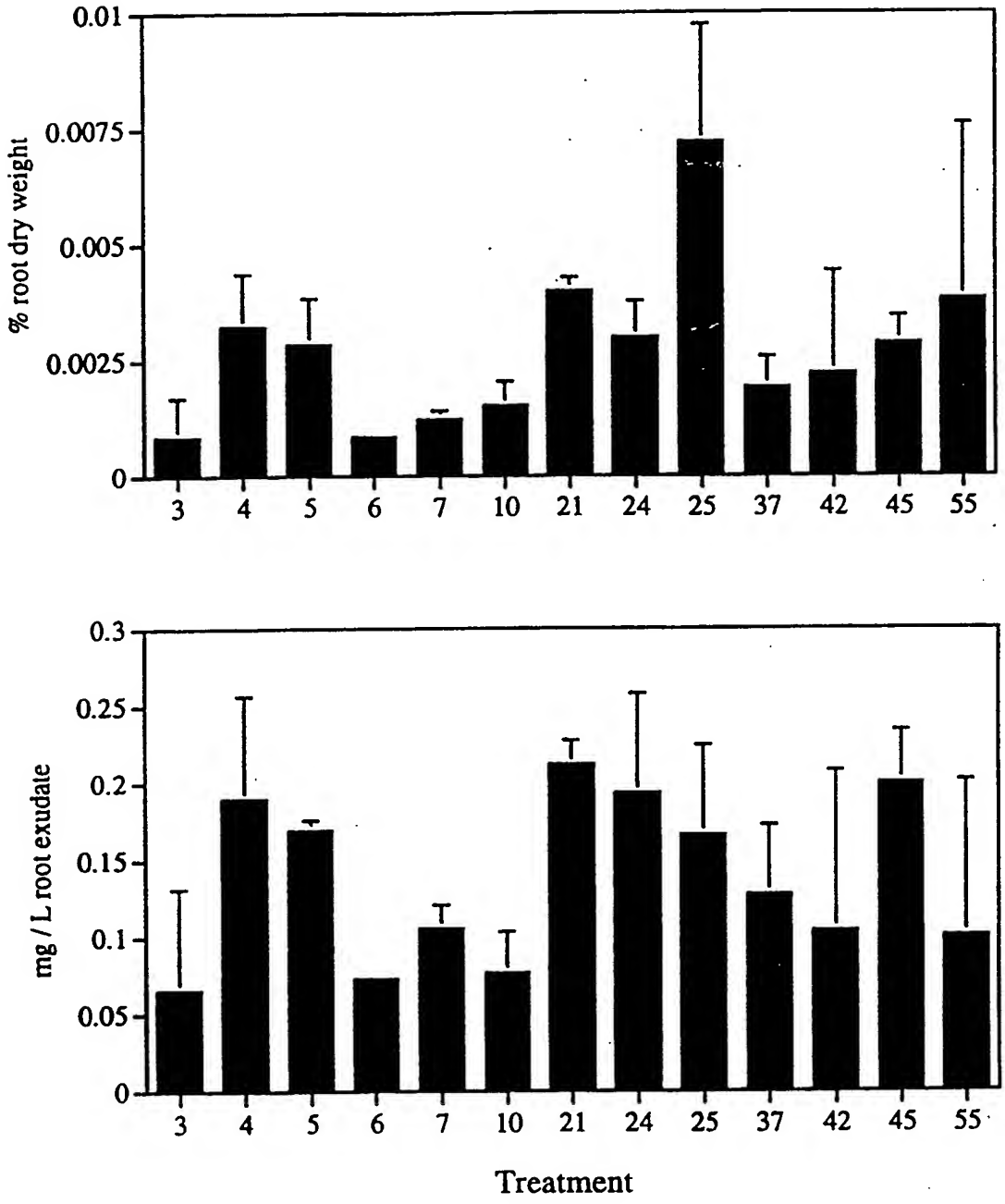

0.01
0.0075
0.005
0.0025
0
% root dry weight
3 4 5 6 7 10 21 24 25 37 42 45 55
0.3
0.25
0.2
0.15
0.1
0.05
0
mg / L root exudate
3 4 5 6 7 10 21 24 25 37 42 45 55
Treatment

Figure 17

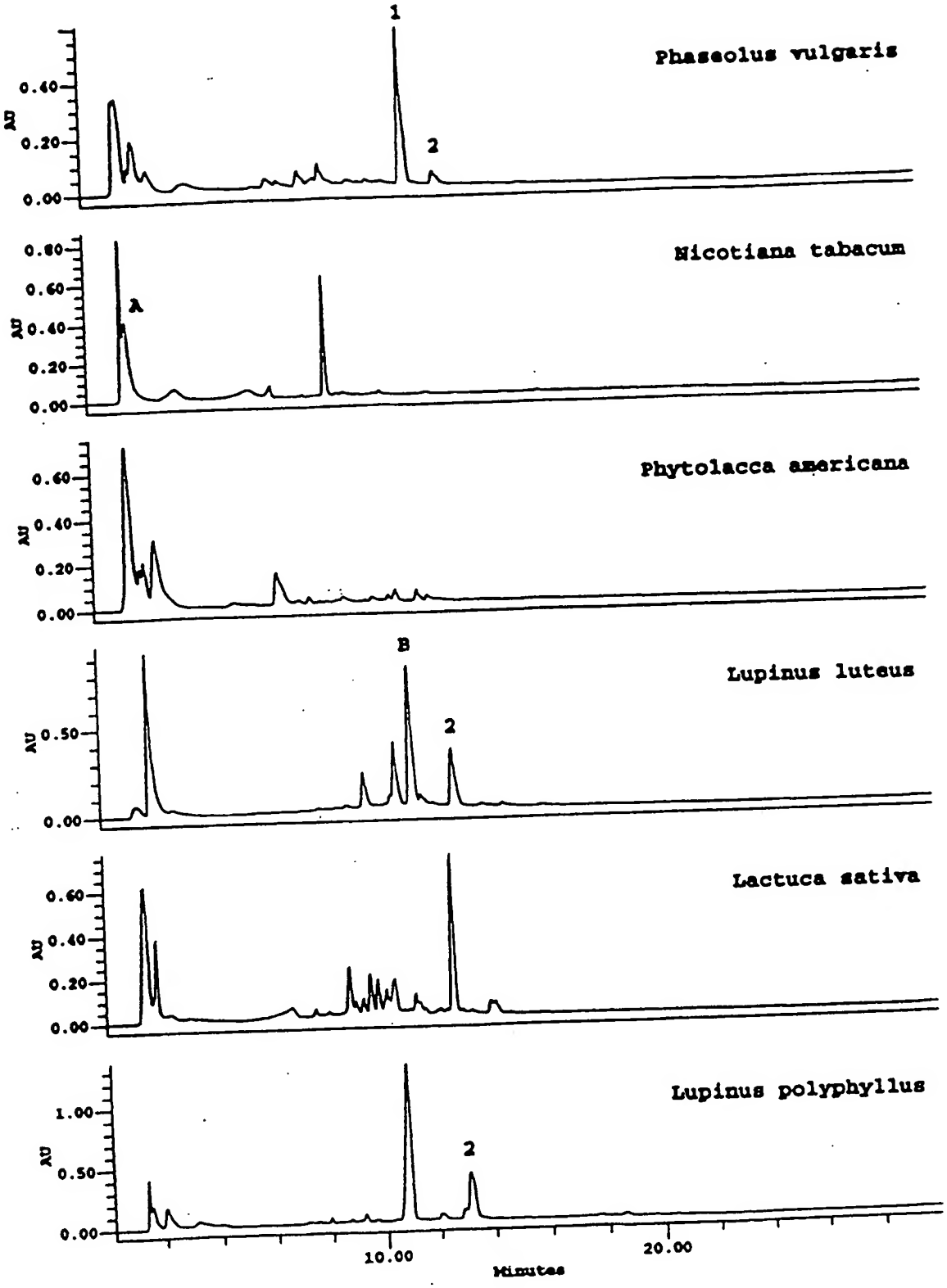

Phaseolus vulgaris
Nicotiana tabacum
Phytolacca americana
Lupinus luteus
Lactuca sativa
Lupinus polyphyllus
AU
Minutes
10.00
20.00
1
2
A
B

Figure 18

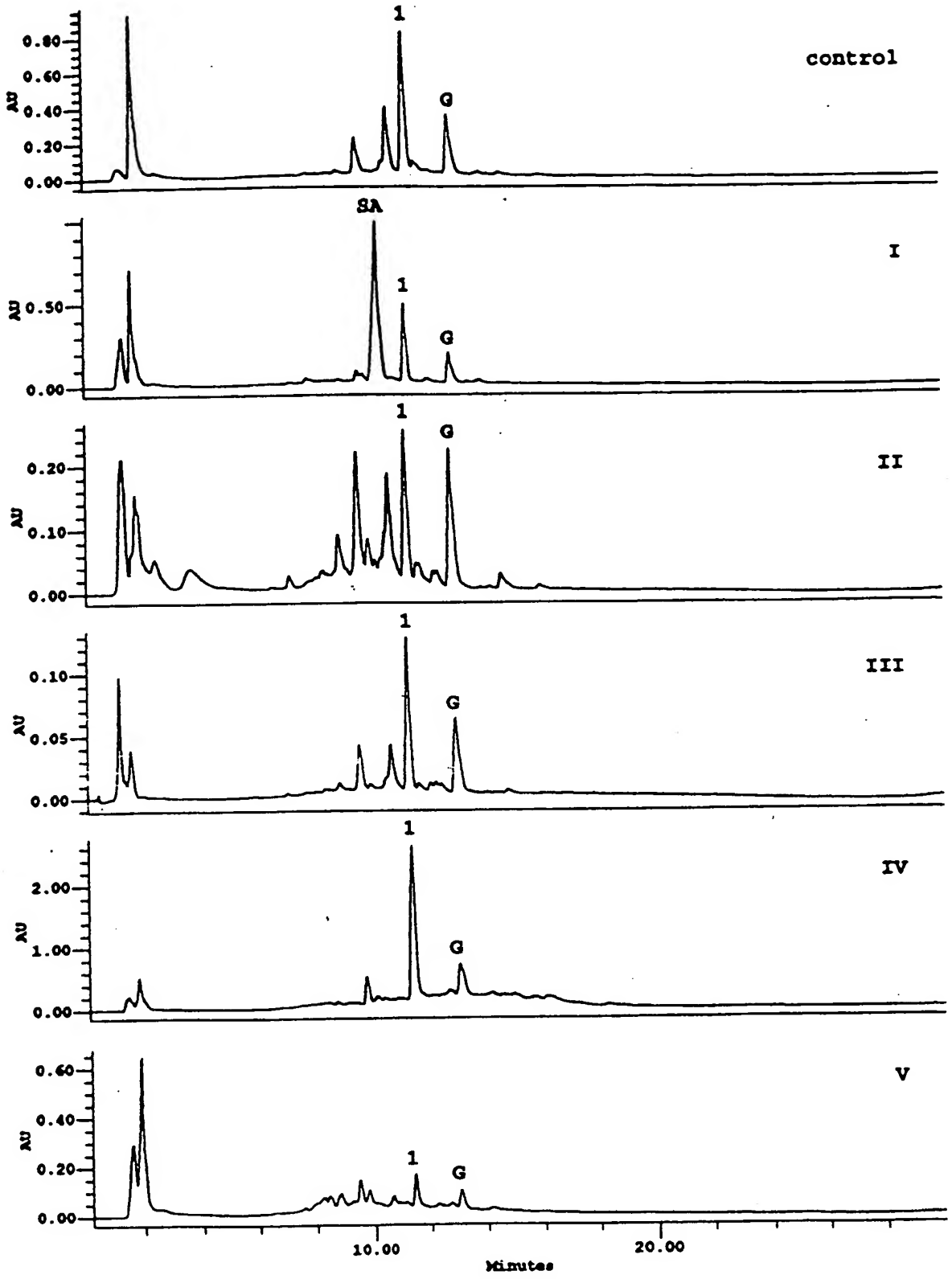
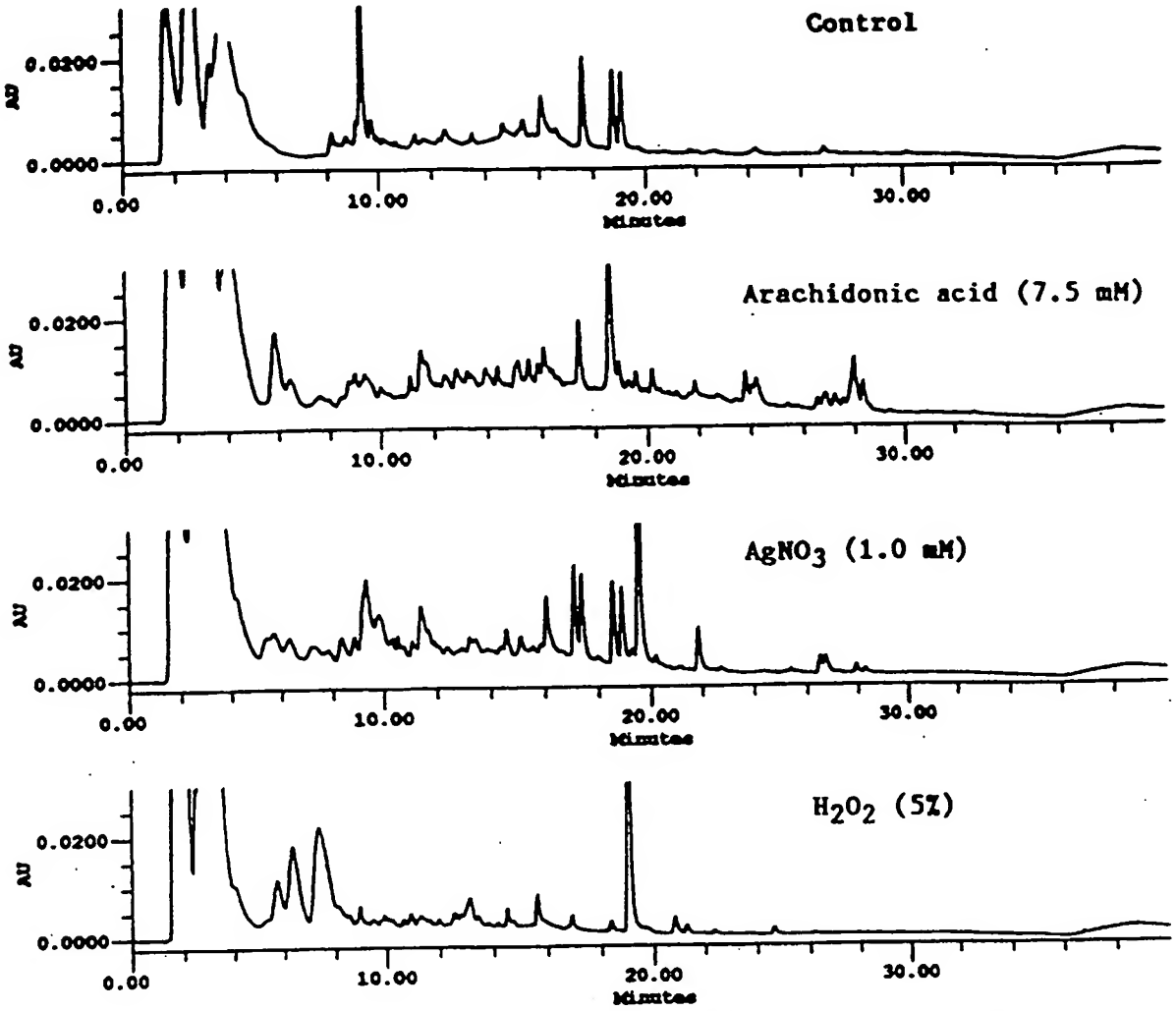
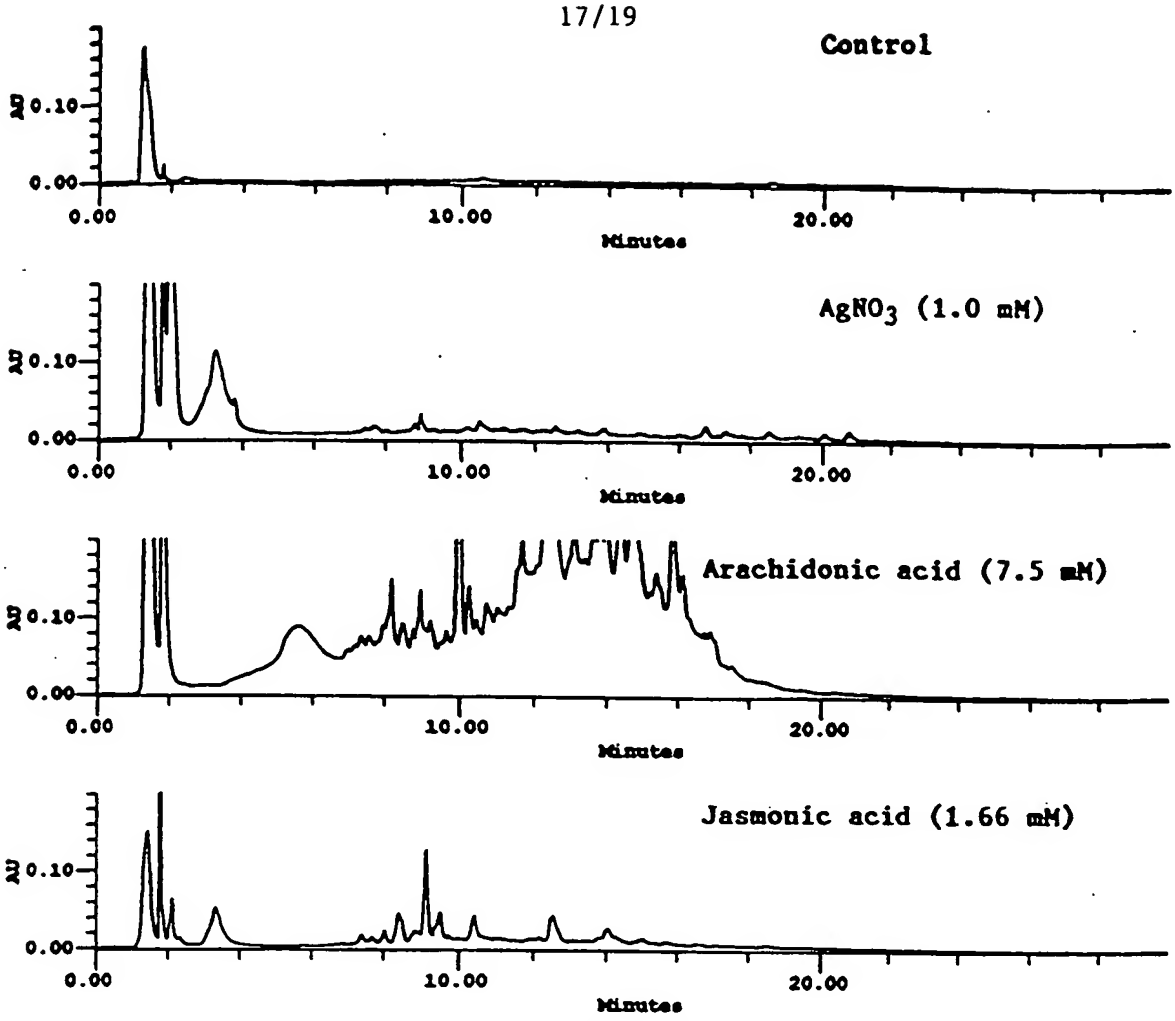

control
I
II
III
IV
V
SA
1
G
AU
0.80
0.60
0.40
0.20
0.00
0.50
0.10
0.05
2.00
1.00
10.00
20.00
Minutes

Figure 19



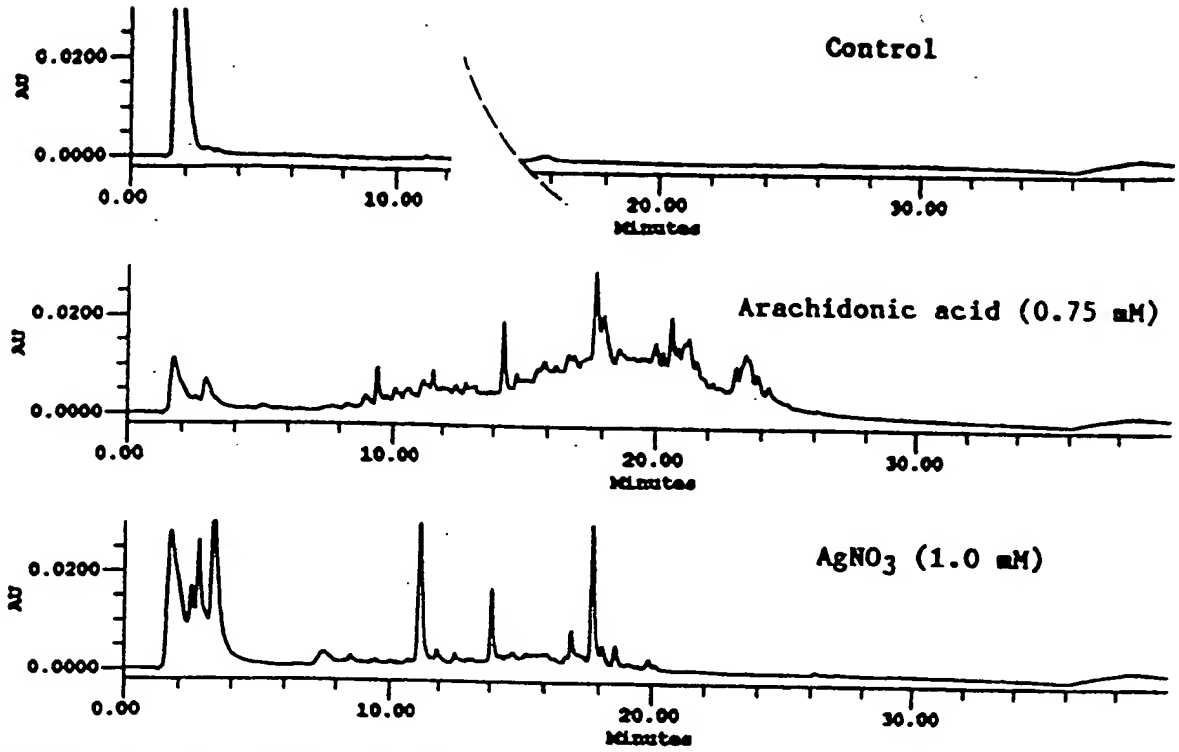
Effect of elicitation on the chemical composition of root exudates of Brassica juncea.
HPLC-profiles with UV detection at 254 nm.

Figure 20



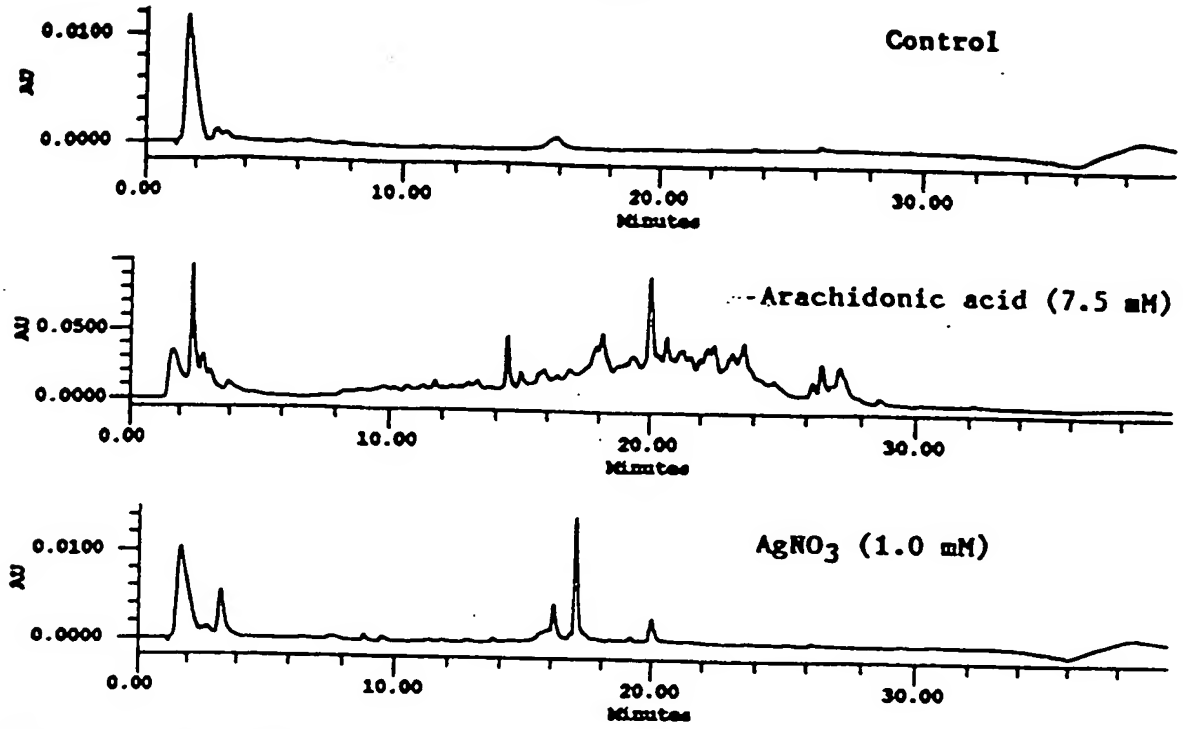
Effect of elicitation on the chemical composition of root exudates of Datura metel.
HPLC-profiles with UV detection at 254 nm.

Figure 21



Effect of elicitation on the chemical composition of root exudates of Lupinus polyphyllus.
HPLC-profiles with UV detection at 254 nm.

Figure 22



Effect of elicitation on the chemical composition of root exudates of Melilotus medicaginoides.
HPLC-profiles with UV detection at 254 nm.

Figure 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17893

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01K 65/00; C12Q 1/02
US CL :424/195.1; 435/29

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/195.1; 435/29

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST USPAT, DERWENT; STN BIOSIS, AGRICOLA.
search terms: leaf exudates, cuticular material, wax, antimicrobial

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHAKRABORTY et al. Accumulation of Antifungal Compounds in Tea Leaf Tissue Infected with Bipolaris carbonum. FOLIA Microbiol. 1994, Vol. 39, No. 5, pages 409-414, entire document.	1-6,10,12, 14,15
Y	STEVENS et al. The Systematic Evolutionary Significance of Exudate Flavonoids in Aeonium. Phytochemistry. 1995, Vol. 39, No. 4, pages 805-813, entire document.	1-15
Y	Database BIOSIS on STN. LIU et al. AN 1996:458578. 'Antibacterial Activity of Flavonoids Against Methicillin-Resistant Staphylococcus aureus'. Dokkyo Journal Of Medical Sciences. 1995, Vol. 22, No. 4, pages 253-261, Abstract.	1-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
21 OCTOBER 1999

Date of mailing of the international search report
17 NOV 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Washington, D.C. 20231
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Authorized officer
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JOYCE BRIDGERS
PARALEGAL SPECIALIST
CHEMICAL MATRIX

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17893

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TYLER V.E. Herbs of Choice: the Therapeutic Use of Phytomedicinals. Pharmaceutical Product Press. New York. 1994, pages 77-78, 96-97, entire document.	1-15
Y	GRIEVE M. A Modern Herbal: The Medicinal, Culinary, Cosmetic And Economic Properties, Cultivation And Folklore Of Herbs, Grasses, Fungi, Shrubs And Trees With All Their Modern Scientific Uses. Barnes and Nobles. New York. 1996, pages 464-465,583-589, 660-661, entire document.	1-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17893

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-15

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-15, drawn to a method of identifying an agent exuded onto a plant leaf.
Group II, claim(s) 16-28, drawn to a method of identifying an agent exuded onto plant roots.
Group III, claim(s) 29-32, drawn to methods for recovering plant-exuded substances and generating a library of plant-exuded substances.

The inventions listed as Groups I, II and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
The methods of inventions of Groups I, II and III are three distinct methods which comprise different active steps as claimed such as assaying/analyzing plant substances obtained from plant leaves with a solvent in the method of Group I or assaying/analyzing plant substances obtained with an aqueous medium from plant roots in the method of Group II or generating library of plant-exuded substances recovered from living plants with an aqueous medium in the method of Group III. Three distinct methods as claimed do not require any special technical feature and they do not fall into anyone of permissible categories set forth in paragraph (b) of the section 37 CFR 1.1475.